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14. ABSTRACT

Prostate cancer is the second leading cause of cancer death among American men. Although radiotherapy (RT) is one of the two curative treatments for prostate cancer patients, approximately 10% of low-risk cancer patients and 30-60% of high-risk prostate cancer patients experience biochemical recurrence within five years, among them 20% die in 10 years. The proposed research is based on the hypothesis that targeting protein arginine methyltransferase 5 (PRMT5) can sensitize primary and recurrent prostate cancer cells to RT. During the first grant period, we have successfully demonstrated that knockdown of PRMT5 or inhibition of PRMT5 by a specific inhibitor can sensitize prostate cancer cells (LNCaP, DU-145 and PC-3) to radiation in vitro. This radiosensitization is likely due to the involvement of PRMT5 in the regulation of the DNA damage response. These results collectively suggest that targeting PRMT5 can sensitize prostate cancer cells to radiation. We are currently isolating stably integrated clones to inducibly knock down PRMT5 for proposed in vivo experiments. We have also successfully isolated 3 radiation-resistant sublines from DU-145 after 40 Gy of fractionated ionizing radiation. These resistant cell sublines along with previously isolated LNCaP radiation-resistant sublines will be used for PRMT5 targeting experiments. In addition, we found that PRMT5 regulates prostate cancer cell growth in an AR-dependent manner, and this effect is likely medicated by epigenetic regulation of AR transcription. We will continue to pursue this novel and exciting finding.

15. SUBJECT TERMS
Prostate cancer, LNCaP, ionizing radiation, PRMT5, DU-145, PC-3, CREB

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Table of Contents

Introduction

Prostate cancer remains the number one cancer diagnosed in men (except skin cancer), and 217,730 new patients were diagnosed and 32,050 died in the US in 2010 [1]. Radiotherapy (RT) is an important primary treatment for old patients with low-risk prostate cancer, the standard primary treatment for high-risk prostate cancer when combined with androgen deprivation therapy (ADT), and the major salvage therapy for local recurrence after surgery [2-6]. In addition, surgery plus adjuvant RT also demonstrates survival benefits when compared with surgery alone [2, 7, 8]. Despite that the majority of patients can be cured by RT, approximately 10% of patients with low-risk cancer and up to 30-60% of patients with high-risk cancer experienced biochemical recurrence within five years after RT, and among them 20% of patients died in 10 years [9-12]. Similar rate of recurrence was observed after surgery [13, 14]. Given that 96% of prostate cancer patients are present as localized cancer in the US [15] and that most recurrent tumors are local recurrence [16], failure in controlling these localized primary and recurrent prostate cancers eventually leads to disease progression and contributes to the majority of prostate cancer deaths. Thus, developing effective primary and salvage RT for prostate cancer patients will have a huge impact on reducing prostate cancer mortality.

Protein arginine methyltransferases (PRMTs) are a family of proteins involved in post-translational modifications of histones and non-histone proteins [17, 18], mRNA splicing, nuclear-cytoplasmic shuttling, DNA damage response, and signal transduction [19]. Recent studies have further demonstrated that PRMT5 is involved in the DNA damage response by epigenectically modulating target gene expression or by regulating the function of proteins that are involved in the DNA damage response [20-22]. However, it remains uninvestigated how PRMT5 is involved in prostate cancer development, progression, and therapeutic responses. Based on the findings in the literature and the preliminary studies, it is hypothesized that radiation-induced or pre-existing PRMT5 overexpression contributes to the resistance of prostate cancer cells to RT in both primary and recurrent prostate cancer. The objective of the proposed research is to determine whether targeting PRMT5 can sensitize primary prostate cancer to RT, and can reprogram therapy-resistant recurrent prostate cancer to therapy-sensitive prostate cancer. Three specific aims are proposed in this project. **Aim 1** will determine that targeting PRMT5 can sensitize prostate cancer cells and prostate cancer xenograft tumors to fractionated ionizing radiation (IR) *in vitro* and in nude mice; **Aim 2** will determine that targeting PRMT5 can sensitize radiation-resistant prostate cancer cell sublines and recurrent xenograft tumors to radiation and chemotherapy *in vitro* and in nude mice; and **Aim 3** is to establish the clinical correlation between the expression level of PRMT5 and radioresistance and tumor recurrence in human prostate cancer patients. Under the support of this award, we have made the following progress during the first grant period (Aug 1, 2012 – July 30, 2013).

Body

Completion of Approved Statement of Work

Task 1. Aim 1: To determine that targeting PRMT5 can sensitize prostate cancer cells and prostate cancer xenograft tumors to radiation *in vitro* and in nude mice (Months 1-18)

1a. Generate lentivirus for making doxycycline-inducible cell lines using LNCP, DU-145 and PC-3 cells (Months 1-6). **Partially completed!**

We have constructed four short-hairpin RNA (shRNA) expressing vectors using the Tet-pLKO-puro vector to knock down PRMT5 and screened for the best one for making lentivirus. As shown in Figure 1A, the PRMT5 shRNA#1577 showed a better knockdown effect after transient induction of the shRNAs for 72 h. This was further confirmed with two different clones of the PRMT5 shRNA#1577 under the condition that LNCaP cells were subjected to 10 Gy of fractionated ionizing radiation (IR). IR-induced CREB phosphorylation (pCREB) was significantly inhibited when PRMT5 was knocked down by two different clones of the same shRNA sequence (Fig. 1B). Thus, we have chosen PRMT5 shRNA#1577 for making lentivirus, which were used for making stable transduction to LNCaP cells. However, we found that these cells could not be maintained for long time and the infected cells were eventually replaced with cells without infection. We are currently selecting for individual clones for establishment of stable cell lines. Once completed, we will use these stable cell lines for proposed *in vivo* experiments.

1b. Perform radiosensitization experiments by using the knockdown cell lines and by using PRMT5 small molecule inhibitor BLL3.3 (months 7-12). **Completed!**

Since radiosensitization experiments do not require long-time maintenance, we have instead performed transient expressing of PRMT5 shRNAs to see if knockdown of PRMT5 increases radiation-induced cell death. As shown in Figure 2, knockdown of PRMT5 by two different clones of the #1577 increased ionizing radiation (IR)-induced cell death. To determine whether knockdown or inhibition of PRMT5 can radiosensitize prostate cancer cells, we have performed clonogenic assays in LNCaP, DU-145 and PC-3 cells. It is worth mentioning that we initially proposed to use MTT and apoptosis assays to determine whether knockdown or inhibition of PRMT5 can radiosensitize prostate cancer cells in the proposal. However, we recently realized that clonogenic assay, rather than MTT or apoptosis assay, is a standard method to determine radiosensitivity of cancer cells. In fact, this assay was also suggested by the Scientist B. Thus, we performed clonogenic assays instead. As shown in Fig. 3A, knockdown of PRMT5 significantly sensitized LNCaP cells to IR. Although DU-145 and PC-3 cells are relatively resistant to radiation, knockdown of PRMT5 also sensitized these cells to IR, albeit to a lesser extent (Fig. 3B and 3C). Further, inhibition of PRMT5 by the inhibitor BLL3.3 similarly sensitized LNCaP cells to IR (Fig. 3D). Thus, our results demonstrated that knockdown or inhibition of PRMT5 can radiosensitize prostate cancer cells to radiation.

1c. Submit animal protocols for approval from Purdue University and USAMRMC. **Completed!**

We have completed the submission and approval of the animal protocols.

1d. Perform in vivo radiosensitization experiments using prostate cancer cell xenograft tumors (LNCaP and DU-145) and analyze data (months 7-12). **Not completed**.

As discussed in subtask 1a, we have found that lentivirus-based stable cell lines are not stable. We are screening for independent clones for proposed *in vivo* experiments. We plan to complete the proposed experiments within the next year.

e. Analyze tumor tissues by immunohistochemistry (months 13-18). **Not started**.

Task 2. Aim 2: To determine that targeting PRMT5 can sensitize recurrent (regrown) xenograft tumors to radiation and chemotherapy (Months 19-36)

2a. Isolate radiation-resistant prostate cancer sublines from DU-145 and PC-3 cells (months 19-24) **Completed!**

We have performed 40 Gy of fractionated IR to DU-145 and PC-3 cells, and waited for cell regrowth. We have successfully isolated 3 radiation-resistant sublines from DU-145. Interestingly, radioresistant PC-3 cells after 40 Gy of fractionated IR remained dormant and no regrowth was observed after more than 3-month observation. This suggests that PC-3 cell cannot be reprogrammed to proliferate.

2b. Perform radiosensitization and chemosensitizatio experiments using radiation-resistant sublines (Months 25-36). **Not started.**

2c. Perform in vivo radiosensitization of recurrent xenograft tumors (Months 19-30). **Not started.**

2d. Analyze tumor tissues by immunohistochemistry (Months 31-36). **Not started.**

Task 3. Aim 3: To establish the clinical correlation between the expression level of PRMT5 and radioresistance and tumor recurrence (Months 1-36)

a. Submit IRB protocols to Purdue University, London Health Science Centre of the University of Ontario and USAMRMC (Months 1-6). **Completed.**

We have completed the submission of IRB protocols and we have received approvals.

b. Retrieve and review specimens for the proposed research (Months 7-12) **Ongoing!**

Dr. Chin and Dr. Moussa at the University of Western Ontario have been retrieving specimens from their archived specimens. Preliminary results show that they have difficulty identifying some primary specimens from those who had recurrent tumors. They are trying to identify exactly how many recurrent specimens only they have and how many pairs of primary and recurrent specimens they have. Based on this information, we will discuss with Dr. Dabao Zhang, who serves as biostatistics collaborator, and decide appropriate approaches to design this clinical correlation study.

3c. Prepare two slides from each specimens for HIS analysis (Months 13-18) **Not started yet!**

3d. Perform IHC analysis and analyze data to establish the clinical correlation between PRMT5 expression and radioresistance and tumor recurrence (Months 19-36) **Not started yet!**

4. Additional accomplishments beyond the Approved SOW

4a. PRMT5 regulates prostate cancer cell growth in AR-dependent manner. During the course of this project, we have accidently found that knockdown of PRMT5 without radiation also slowed down cell growth in LNCaP cells (Fig. 4A). Interestingly, this effect seems to be dependent on the androgen receptor (AR) status, as knockdown of PRMT5 did not affect cell growth in DU-145 and PC-3 cells, both of which did not express detectable level of AR (Fig. 4B-C). Consistent with a role of PRMT5 in regulation of LNCaP cell growth, knockdown of PRMT5 also inhibited colony formation in soft agar assays (Fig. 4D). These results suggest that PRMT5 expression may promote prostate cancer cell growth by controlling the expression of AR, a driver of prostate cancer growth. This is a very important and exciting finding given that RT plus ADT is the standard treatment for high-risk prostate cancer patients and ADT is considered a radiosensitization agent in this treatment regimen. This novel finding is also consistent with our overall hypothesis that targeting PRMT5 may sensitize prostate cancer to RT, and suggests that targeting PRMT5 is a double-edged sword for prostate cancer RT.

4b. PRMT5 regulates transcription of AR. Since AR is important for cell growth via transcriptional regulation of its target genes, we examined whether knockdown of PRMT5 has any effect on the AR activity. As shown in Fig. 5A, knockdown of PRMT5 inhibited androgen-induced AR activity in a PSA-luciferase reporter gene assay, which is confirmed by the down-regulation of PSA and AR expression (Fig. 5B), suggesting that the inhibition of AR activity by PRMT5 knockdown is likely due to the down-regulation of AR and PSA expression. Because PRMT5 is an epigenetic regulator, we next examined whether PRMT5 regulates expression of AR at the transcriptional level using quantitative real-time PCR, and found that knockdown of PRMT5 significantly down-regulated the transcription of AR (Fig. 5C). Taken together, these results suggest that PRMT5 may epigenetically regulate AR transcription. We will continue to explore this to determine whether PRMT5 epigenetically regulates AR transcription.

4c. PRMT5 is involved in DNA repair. As suggested by the Scientist B, we have also examined whether PRMT5 knockdown affects damage response. We have found that knockdown of PRMT5 significantly increased the staining of γH2AX (Fig. 6A) and the expression level (Fig 6B), confirming that PRMT5 is involved in the regulation of the DNA damage response. This additional accomplishment is related to *Task 1b*.

Key Research Accomplishments

- Constructed 4 PRMT5 shRNAs plasmids and identified the #1577 as the best one to knock down PRMT5.
- Demonstrated that knockdown of PRMT5 can sensitize LNCaP, DU-145 and PC-3 cells to radiation in clonogenic assays.
- Demonstrated that inhibition of PRMT5 can also sensitize LNCaP cells to radiation in clonogenic assays.
- Isolated 3 radiation-resistant sublines from DU-145 after subjecting to 40 Gy of fractionated IR.
- Discovered that PRMT5 also regulates the growth of prostate cancer cells in an AR-dependent manner.
- Discovered that PRMT5 regulates transcription of AR.
- Confirmed that PRMT5 is involved in the DNA damage response in LNCaP cells.

Reportable Outcomes

1. Manuscripts, abstracts, presentations
   (1) Publication of research results in Prostate Cancer Research area
   Hsu, C.C. and Hu, C.D. Transcriptional activity of –Jun is critical for the suppression of AR function. Mol Cell Endocrinol, 372:12-22 (2012)
   (2) Meeting attendance
   Bimolecular fluorescence complementation (BiFC): Current Status and Future Perspectives
   Drug Discovery Chemistry in San Diego: Sixth Annual Protein-Protein Interactions (Targeting PPI for Therapeutic Interventions), April 17-18, 2013
   (3) Invited Seminars relevant to the project
      a. Neuroendocrine differentiation (NED): A therapeutic challenge in prostate cancer management
      Place: Tongji Hospital, Huazhong University of Science and Technology
      Date: February 2, 2013
      b. Neuroendocrine differentiation (NED) in prostate cancer cells: From basic science to clinical practice
      Place: Jinan University School of Medicine
      Date: May 17, 2013
      c. Neuroendocrine differentiation (NED) in prostate cancer cells: From bench to bedside
      Place: Union Hospital
      Date: May 17, 2013
      d. Impact of neuroendocrine differentiation in prostate cancer radiotherapy
      Place: Hefei Chinese Academy of Sciences Cancer Hospital
      Date: May 24, 2013
2. Licenses applied for and/or issued
   None
3. Degrees obtained that are partially supported by this award
   Christopher Suarez, Ph.D. awarded in December, 2012
   Chih-chao Hsu, Ph.D. awarded in December, 2012
4. Development of cell lines, tissue or serum repositories
   We have isolated 3 radiation resistant clones from DU-145 that were subjected 40 Gy of fractionated IR. These clones will be useful for molecular mechanism study and for development of novel therapeutics.
5. Funding applied for based on work supported by this award
   Title: Targeting neuroendocrine differentiation for prostate cancer radiotherapy
   Agency: 2012 DOD Prostate Cancer Research Program (PCRP)
   Total Cost: $559,055
   Period: 08/01/2013– 07/30/2016
   Outcome: Pending for funding

   Title: Impact of neuroendocrine differentiation in prostate cancer radiotherapy
   Agency: National Cancer Institute
   Period: 07/01/2014-06/30/2019

Total budget requested: $3,154,076
Outcome: Pending for review

6. Employment or research opportunities applied for and/or received based on experience/training support by this award
   Christopher Suarez: conducting his postdoctoral research on prostate cancer neuroendocrine differentiation at University of Notre Dam
   Chih-chao Hsu: conducting his postdoctoral research on cancer research at Baylor College of Medicine

Conclusion

Under the support of this prostate cancer idea development award, we have successfully constructed several shRNA plasmids to knock down PRMT5. Using one of the best knockdown constructs, we have demonstrated that knockdown of PRMT5 can sensitize prostate cancer cells (LNCaP, DU-145 and PC-3) to radiation *in vitro*. Consistent with this, inhibition of PRMT5 by a specific inhibitor BLL3.3 also sensitized LNCaP cells to radiation. We also observed that PRMT5 knockdown increased fractionated IR-induced staining and the expression level of γH2AX in LNCaP cells, an indicator of double-stranded breaks, confirming that PRMT5 is involved in the regulation of the DNA damage response reported by others. We are currently isolating stably integrated clones for proposed *in vivo* experiments. During the course of performing PRMT5 knockdown and radiosensitization experiments, we additionally found that PRMT5 regulates prostate cancer cell growth in an AR-dependent manner. Interestingly, this effect appears to be regulated by the transcription of AR. As PRMT5 is an epigenetic regulator, our novel finding suggests that PRMT5 may epigenetically regulate AR expression. Given that radiotherapy combined with androgen deprivation therapy is the standard treatment for high-risk prostate cancer, we will continue to explore whether PRMT5 is an epigenetic regulator of AR during the next grant period. If so, targeting PRMT5 is a double-egged sword for prostate cancer radiosesitization. We will continue with proposed experiments in Aim 2 and Aim 3.

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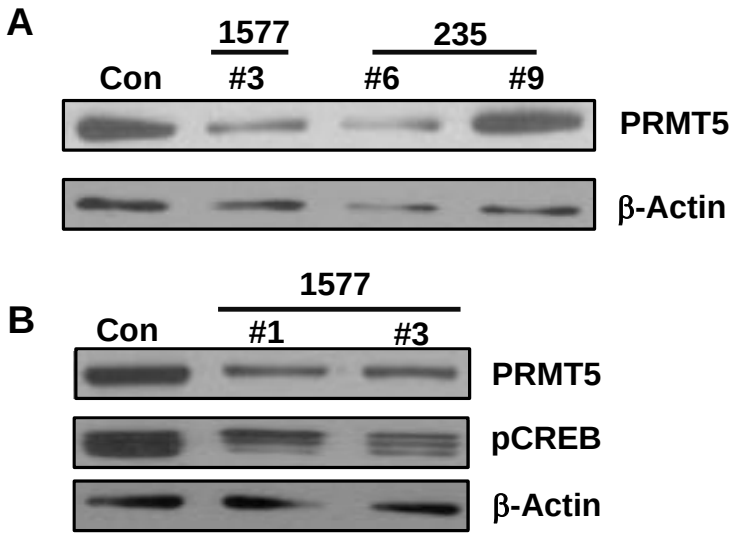

Figure 1. Selection of PRMT5 knockdown constructs. A. Shown are effect of two representative PRMT5 shRNA constructs (sequence #1577 and 235) and three different clones on PRMT5 expression. LNCaP cells were transfected with the vector control (Con) or the two PRMT5 shRNA constructs (#1577 and #235) with Fugene HD, and cells were harvested at 72 h post-transfection for Western blotting analysis of PRMT5 expression. **B**. Effect of PRMT5 knockdown on radiation-induced CREB activation in LNCaP cells. Two different clones (#1 and #3) of the PRMT5 shRNA#1577 were transiently transfected into LNCaP cells, followed by fractionated ionizing radiation (IR) for a total dose of 10 Gy (2 Gy/day). Cells were then harvested to determine the expression of PRMT5 and the activation of CREB (pCREB).

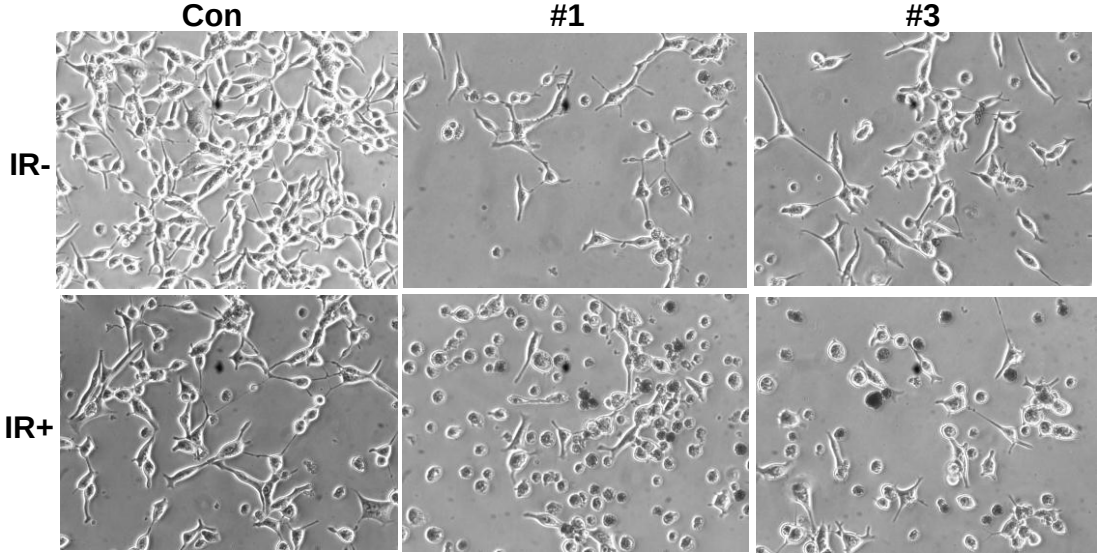

Figure 2. Knockdown of PRMT5 increases IR-induced cell death. LNCaP cells were transfected with the PRMT5 shRNA#1577 (clone #1 and #3) or the vector control (Con) for 48 h, followed by IR (2 Gy/day) for three days (IR+). Similar control experiment was performed without irradiation (IR-). Phase contrast images shown were taken 24 h after the third irradiation.

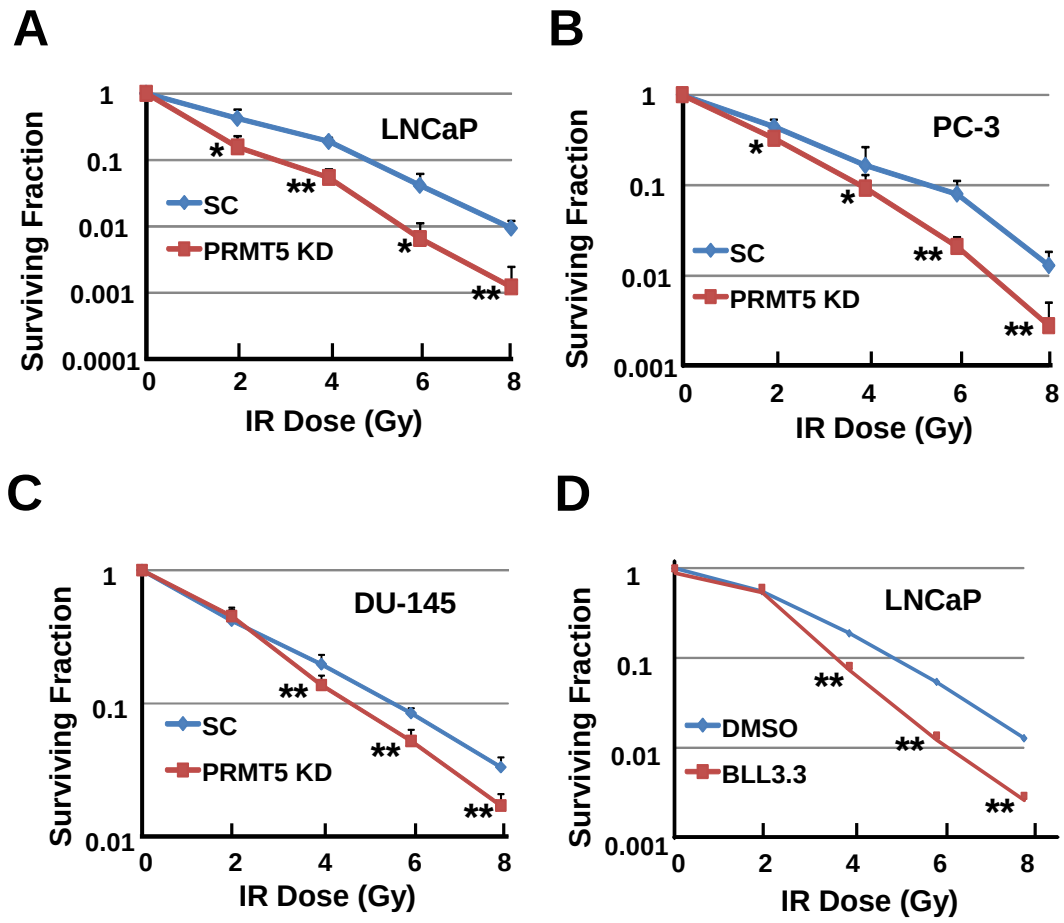

Figure 3. Knockdown or inhibition of PRMT5 sensitizes prostate cancer cells to radiation. **A-C**. The indicated prostate cancer cells were transiently transfected with the PRMT5 shRNA#1577 for 48 h, and then subjected to the indicated dose of IR. Cells were immediately trypsinized and counted, and various numbers of cells were seeded in 6-well plates for the formation of colonies for 14 days. The number of colony was counted and surviving fraction was calculated. **D**. LNCaP cells were treated with 10 μM of BLL3.3 for 48 h, followed by similar procedures for the clonogenic assays described above. Results are from three independent experiments.

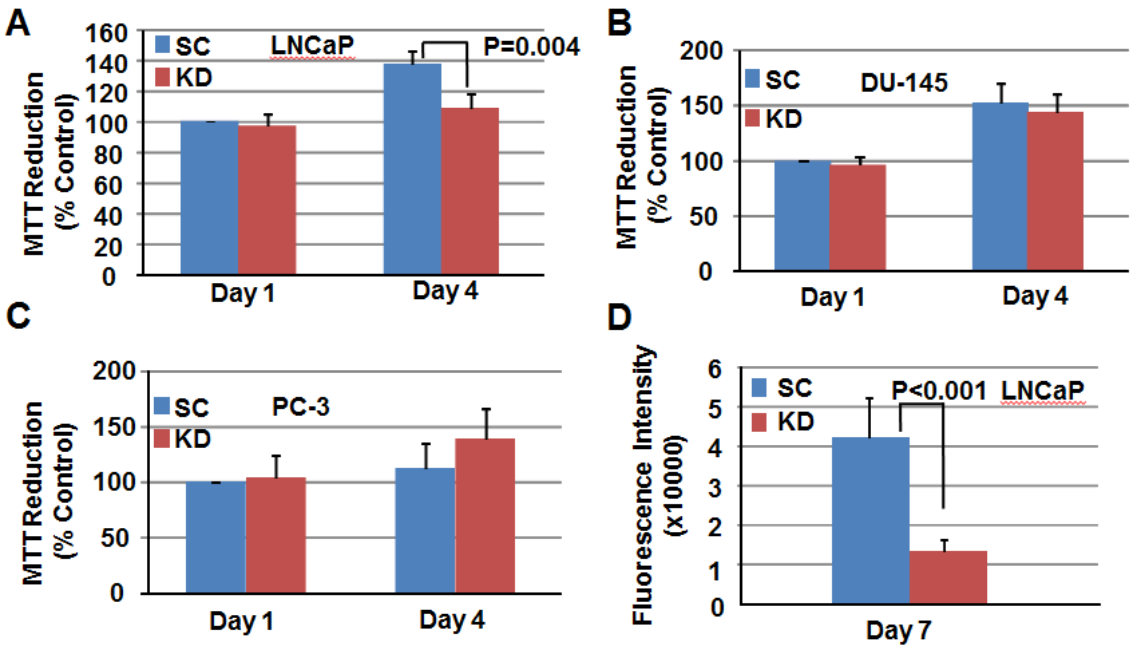

Figure 4. PRMT5 regulates prostate cancer cell growth in an AR-dependent manner. A-C. The indicated prostate cancer cells were transiently transfected with the PRMT5 shRNA#1577 (KD) or the scrambled control (SC). Cell viability was determined at day 1 and day 4 after the transfection. **D**. LNCaP cells were similarly transfected with the knockdown construct or the control for 48 h, and then used for the 96-well Almar Blue soft agar assays. Results are from three independent experiments.

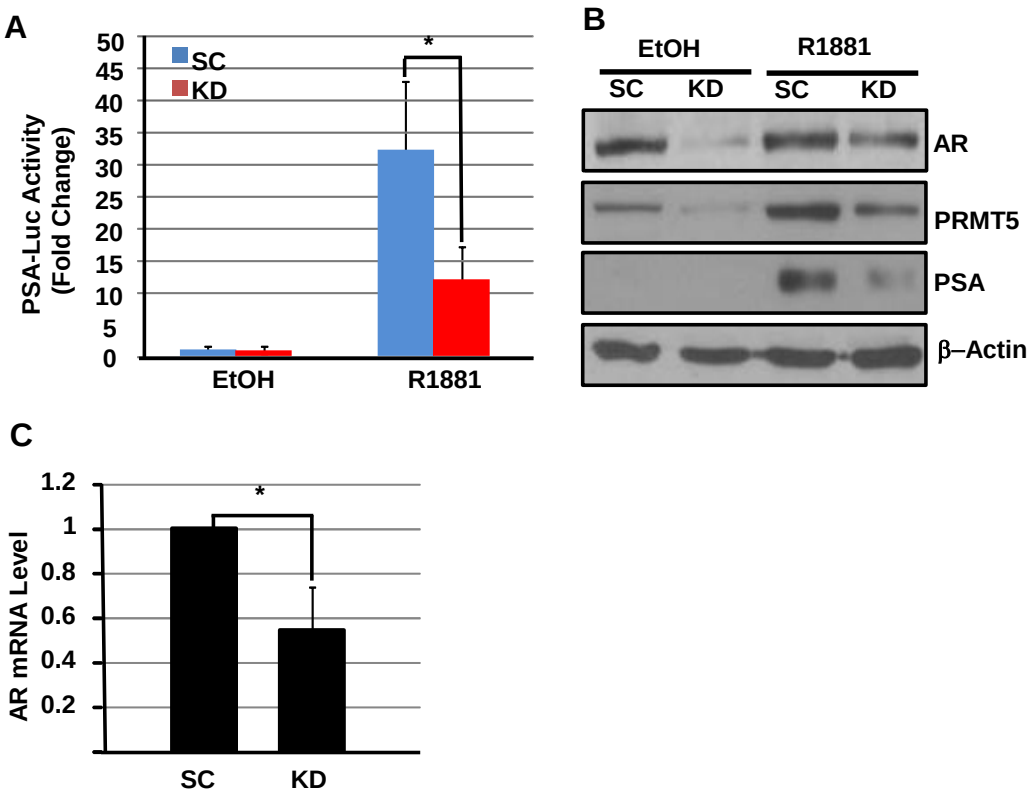

Figure 5. PRMT5 regulates AR expression. A. LNCaP cells were transiently transfected the PRMT5 shRNA#1577 (KD) or the scrambled control (SC) along with the PSA-Luc reporter plasmid as well as the Renila plasmid for 48 h, and then treated with 1 nM of R1881 or ethanol (EtOH) for 24 h. The luciferase activity was assayed using the Dual Luciferase Assay Kit (Promega). **B**. Cell lysate from the same experiment in A was used for Western blotting of AR, PRMT5 and PSA. **C**. LNCaP cells were transiently transfected with the PRMT5 shRNA#1577 (KD) or the scrambled control (SC) for 72 h, and then qPCR was performed to determine the expression of AR mRNA.

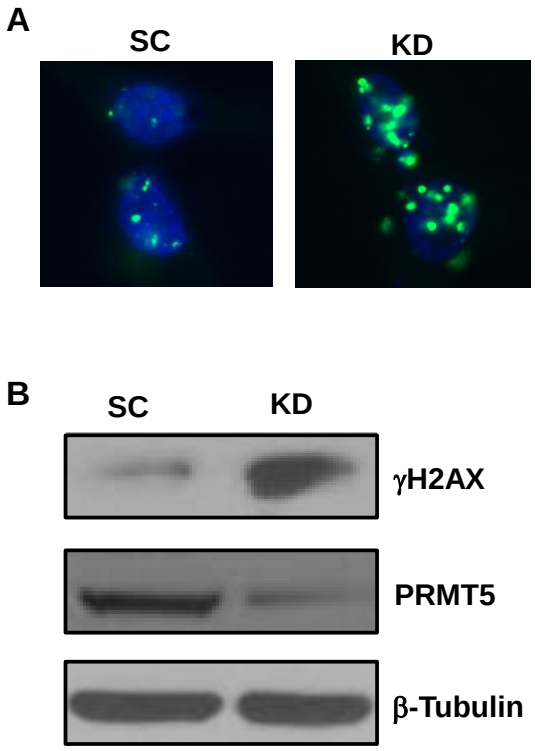

Figure 6. PRMT5 regulates the DNA damage response in prostate cancer cells. LNCaP cells were transiently transfected with the PRMT5 shRNA#1577 (KD) or the scrambled control (SC) for 24 h, followed by fractionated IR (2 Gy/day x 5). **A**. Cells were immunostained with anti-γH2AX (Green) after the treatment. Nucleus was stained with DAPI (Blue). **B**. Cells were harvested for Western blotting analysis of γH2AX after the treatment.

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Transcriptional activity of c-Jun is critical for the suppression of AR function

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ABSTRACT

Androgen receptor (AR) signaling plays a pivotal role in growth and survival of prostate cancer cells. c-Jun is an important member of the activator protein 1 (AP-1) family and was shown to interact with AR. However, the role of c-Jun in AR signaling remains controversial, with being a coactivator or a corepressor reported. Here, utilizing multiple approaches, we show that c-Jun efficiently inhibits AR activity and the growth of prostate cancer cells. Overexpression of c-Jun inhibits not only the activities of various androgen-responsive promoters but also the transcripts of multiple AR target genes. Interestingly, long-term c-Jun overexpression also down-regulates AR expression at both the protein and mRNA levels. Molecular analysis suggests that c-Jun inhibits AR transactivation potential via an unknown target gene. The inhibition of AR by c-Jun occurs in both hormone naïve and castration-resistant prostate cancer cells. Our results unravel a novel mechanism by which c-Jun antagonizes the AR signaling.

1. Introduction

Prostate cancer represents the most common non-cutaneous human cancer and is the second leading cause of cancer deaths among men in the US (Jemal et al., 2010). Like normal prostate gland, the proliferation and survival of prostate cancer cells rely on androgens, which signal through the androgen receptor (AR). Thus, androgen ablation therapy, also known as hormone therapy, is the most effective way to control advanced prostate cancer (Salesi et al., 2005). Despite the success of hormone therapy, most tumors eventually relapse and develop into castration-resistant prostate cancer (CRPC) due to the aberrant restoration of AR activity (Feldman and Feldman, 2001). Interestingly, numerous studies have showed that AR signaling axis remains essential for the development and maintenance of CRPC (Chen et al., 2004; Gao et al., 2006; Snoek et al., 2009; Yuan et al., 2006; Zegarra-Moro et al., 2002).

Similar to other steroid hormone receptors, AR is composed of an N-terminal domain (NTD) which contains a major activation domain, AF-1, a DNA-binding domain (DBD), a hinge region and a C-terminal ligand binding domain (LBD) containing a weak activation domain, AF-2 (Dehm and Tindall, 2007). Unliganded AR is sequestered in the cytoplasm by heat shock proteins (Marivoet et al., 1992). Upon binding to testosterone or dihydrotestosterone (DHT), the two major physiology androgens, AR dissociates from heat shock proteins and translocates to the nucleus where it functions as a transcription factor by binding as a homodimer to the androgen-response element (ARE) in the promoter and/or enhancer regions of target genes. c-Jun is a basic region leucine zipper (bZIP) transcription factor and is an important member of the activator protein 1 (AP-1) family (Vogt, 2001). The basic region of c-Jun is required for DNA binding while the leucine zipper enables c-Jun to form a homodimer or a heterodimer with other AP-1 members, such as Fos and activating transcription factor 2 (ATF2). Depending on the dimerization partner, c-Jun/AP-1 complex binds to TPA-responsive elements (TREs) or cyclic AMP-responsive elements (CREs) in the promoter region of target genes that are involved in several cellular responses including proliferation, apoptosis and differentiation (Eferl and Wagner, 2003). Phosphorylation at residues Ser-63 and Ser-73 by c-Jun N-terminal kinases (JNKs) was shown to enhance the transactivation activity of c-Jun (Karin, 1995; Smeal et al., 1991).

Many molecular and genetic studies have provided evidence that AP-1 activity may also be implicated in the development and progression of prostate cancer. The expression of JunB and Fos was found to be up-regulated in primary prostate tumors but down-regulated in metastatic samples (Chandran et al., 2007). Conversely, Ouyang et al. (2008) reported that while both c-Jun and Fos are up-regulated in metastatic prostate cancer, only high c-Jun expression is associated with poor prognosis. However, in the same report, it was found that only few cases (3–4%) of prostate cancer showed high expression of the AP-1 proteins. On the

Abbreviations: AP-1, activator protein 1; CRPC, castration-resistant prostate cancer; DBD, DNA-binding domain; ARE, androgen-response element; DHT, dihydrotestosterone; bZIP, basic region leucine zipper; TRE, TPA-responsive elements; CRE, cyclic AMP-responsive elements; Dox, doxycycline; TMPRSS2, transmembrane protease, serine 2; CBP, CREB binding protein.

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other hand, it has also been observed that some AP-1 proteins are also down-regulated in a subset of prostate cancer patients. In fact, Edwards et al. (2004) found that while 16% of CRPC patients showed c-Jun up-regulation, 20% of CRPC patients exhibited c-Jun down-regulation. Moreover, Tamura et al. (2007) showed that transcripts of both c-Jun and Fos were down-regulated in CRPC. Although these studies examined the expression level of AP-1 proteins in prostate cancer tissues, it remains unclear whether and how their transcriptional activity correlates to the development and progression of prostate cancer.

In addition to functioning as an AP-1 transcription factor, AP-1 proteins also interact with other family of transcription factors such as NF-kappaB (Fujioka et al., 2004; Shyu et al., 2008), NFAT (Macian et al., 2000) and nuclear hormone receptors (Herrlich, 2001; Lamph, 1991). In fact, several studies have suggested that c-Jun may physically interact with AR and modulate the AR activity. Sato et al. (1997) reported that c-Jun can interact with the DNA-binding domain of AR via its leucine zipper region to inhibit the DNA-binding as well as the transcriptional activity of AR. Recently, Mulholland and the coworkers proposed that the up-regulation of c-Jun in PTEN null murine prostate cancer cells contributes to CRPC progression by suppressing AR function and thus reducing the androgen-dependence (Mulholland et al., 2011). Conversely, it was also shown that c-Jun functions as an AR coactivator by enhancing the intramolecular interaction between amino and carboxyl termini of AR (Bubulya et al., 2001, 2000, 1996; Chen et al., 2006; Shemshedini et al., 1991; Wise et al., 1998). Despite the controversy of being an AR coactivator or corepressor, it remains unclear if transcriptional activity of c-Jun is involved in these regulations. Because of the critical role of AR in prostate cancer development and progression and because of the potential regulatory role of AP-1 in the AR signaling, we took a different approach to evaluate the impact of the transcriptional activity of c-Jun on the AR signaling. We found that the DNA binding and transcriptional activities of c-Jun, rather than its physical interaction with AR, are required for the maximal inhibition of the AR signaling. Taken together, our results suggest that an unknown target gene of c-Jun is required for the inhibition of the AR activity and future identification of such a target gene will provide new insight into the regulatory role of AP-1 in the AR signaling and prostate cancer development and progression.

2. Materials and methods

2.1. Antibodies

Polyclonal anti-AR antibody (sc-816) and monoclonal anti-phospho-c-Jun antibody (sc-822) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-PSA antibody (1984-1) was purchased from Epitomics (Burlingame, CA). Monoclonal Anti-β-Tubulin (T0198) and anti-Flag M2 (F3165) antibodies were from sigma. Monoclonal Anti-Human PARP antibody (4C10-5) was purchased from BD Biosciences (San Diego, CA).

2.2. Cell culture

LNCaP and COS-1 cells were purchased from American Type Culture Collection (Manassas, VA). C4-2 cells were obtained from the University of Texas MD Anderson Cancer Center (Houston, TX). LNCaP cells were cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) and C4-2 cells were maintained in T-medium with 10% FBS (Gleave et al., 1991; Wu et al., 1994). COS-1 cells were cultured in DMEM medium supplemented with 5% FBS. For androgen treatment, LNCaP or C4-2 cells were cultured in phenol red-free RPMI1640 with 10% charcoal/dextran-stripped FBS (designated androgen-depleted medium) for 24 h before transient transfection or doxycycline (Dox) induction of c-Jun expression for another 24 h. Cells were then treated with 10 nM R1881 for 24 h. To determine the effect of c-Jun on the expression of endogenous AR-regulated genes, cells were cultured in regular medium (RPMI 1640 with 10% FBS for LNCaP cells; T-medium with 10% FBS for C4-2 cells) before induction of c-Jun expression for indicated periods of time.

2.3. Plasmids

Human c-Jun was amplified from a cDNA library originated from HEK 293T cells and was cloned in frame into the EcoRI/KpnI site of pFlag-CMV2 (sigma). Plasmids encoding c-Jun63A/73A, c-JunΔLZ (c-JunΔ280-317), c-Jun A→D^{265} In265 and TAM67 (c-JunΔ3-122) were generated by PCR or ligation PCR (Ali and Steinkasserer, 1995). To clone pLVX-Tight-Puro-Flag-c-Jun, the cDNA encoding Flag-c-Jun was amplified from pFlag-c-Jun by primer sets: BamHI-Kozak-Flag F (5′-CGG GAT CCG CCG CCA CCA TGG ACT ACA AAG ACG ATG ACG-3′) and c-Jun-stop-EcoRV R (5′-GGG ATA TCT TAA AAT GTT TGC AAC TGC TGC G-3′). The amplified cDNA was then cloned into BamHI and Klenow-blunted EcoRI sites of pLVX-Tight-Puro (Clontech). Similar strategy was used to clone all other Flag-c-Jun mutants into pLVX-Tight-Puro. All constructs generated by the PCR-based method were confirmed by DNA sequencing. For generation of c-Jun short hairpin RNA (shRNA) plasmid, annealed oligonucleotides (The RNAi Consortium TRCN0000010366) targeting TAGTACTCCTTAAGAACACAA in the 3′ untranslated region of c-Jun were cloned into pLKO-Tet-On (Wiederschain et al., 2009) to produce pLKO-Tet-On-c-JunKD.

2.4. Generation of Dox-inducible stable cell lines

LNCaP or C4-2 stable cell lines with inducible wild-type or mutant c-Jun were generated by Lenti-X Tet-On Advanced Inducible Expression System (Clontech) according to the manufacturer's protocol with the following modifications. Cells were first infected with viral particles constitutively expressing rtTA-advanced protein (a mutant Tetracycline-repressor). To generate viral particles, HEK 293T cells cultured in 10-cm culture dish were cotransfected with 2 μg of pLVX-Tet-On, 1.5 μg of pHR′-CMV-ΔR8.20vpr, and 0.5 μg of pHR′-CMV-VSV-G using Fugene HD reagent (Roche Applied Science). The supernatant containing viruses was harvested 2 days post-transfection and was then filtered through a 0.45 μm filter to remove cell debris. Infection was carried out by applying 4 ml of viral supernatant to LNCaP or C4-2 cells cultured in 11 ml complete medium. Polybrene was added at a final concentration of 8 μg/ml to facilitate infection. Two days after infection, cells were selected with 500 μg/ml G418 for more than one week. The cells stably expressing rtTA-advanced protein (LNCaP- or C4-2-rtTA cells) were then transduced with lentiviral particles packaged with pLVX-Tight-Puro-Flag-c-Jun (wild type or mutants) using similar procedures as described above. Following transduction for 2 days, cells were then selected with 2 μg/ml puromycin for 2 additional days. To generate LNCaP cells with inducible c-Jun knockdown, cells were infected with lentiviral particles packaged with pLKO-Tet-On-c-JunKD followed by puromycin selection using similar methods described above.

2.5. Luciferase reporter gene assay

LNCaP or C4-2 cells were trypsinized and washed with phosphate-buffered saline (PBS) once, followed by seeding in a 12 well plate at a density of 1×10^5 cells/well in androgen-depleted medium. Twenty-four hours later, cells were transfected with 0.5 μg of an androgen-responsive luciferase reporter construct, 100 ng of

pRL-TK (Promega) along with indicated amount of plasmids encoding wild type or mutant c-Jun using Fugene HD transfection reagent (Promega). Empty vector (pFlag) was added to keep the same amount of transfected DNA per well. At 24 h post-transfection, cells were treated with ethanol (vehicle control) or 10 nM R1881 and then incubated for another 24 h. Firefly and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay kit (Promega) as previously described (Hsu and Hu, 2012). Relative luciferase activity (Firefly/*Renilla*) was shown as mean ± S.E. from at least three independent experiments performed in duplicate. To analyze the expression level of the exogenous c-Jun proteins (Figs. 1E and 3E), 60 μg of cell lysate were precipitated by incubating with 4 volume of acetone at −20 °C overnight. The protein was then pelleted by centrifugation at 14,000 rpm for 20 min at 4 °C. The pelleted protein was air dried, followed by resuspension in 2X SDS sample buffer and Western blot analysis using anti-Flag-antibody.

2.6. Quantitative real-time PCR (qRT-PCR)

RNA was isolated from LNCaP cell lines using TRIzol (Life Technologies). One microgram of the total RNA was reverse transcribed using random primers (100 ng) and MMLV reverse transcriptase (Promega). qRT-PCR was conducted using Brilliant SYBER Green QPCR Master Mix (Stratagene) in a Mx3000P qPCR system (Stratagene). Expression levels of AR-regulated genes were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results are presented as mean ± S.E. from at least three independent experiments performed in duplicate. The sequences of primers used are listed below. PSA F: 5′-TTG TCT TCC TCA CCC TGT CC-3′; PSA R: 5′-GAG AGG CCA CAA GCA CCT G-3′; KLK2 F: 5′- TGG CTG TGT ACA GTC ATG GA-3′; KLK2 R: 5′-CCT GTG TCT TCA GGC TCA AA-3′; TMPRSS2 F: 5′-AGG TGC ATC CGG CTC AGT A-3′; TMPRSS2 R: 5′-GGG TCA AGG TGA TGC ACA GT-3′; PCDH11 F: 5′-GCG TTT CTG ACT GTG GCT ATC-3′; PCDH11 R: 5′-GGA AGG GGA ATG GAA TTT TG-3′; UGT2B15 F: 5′-TCA AAT

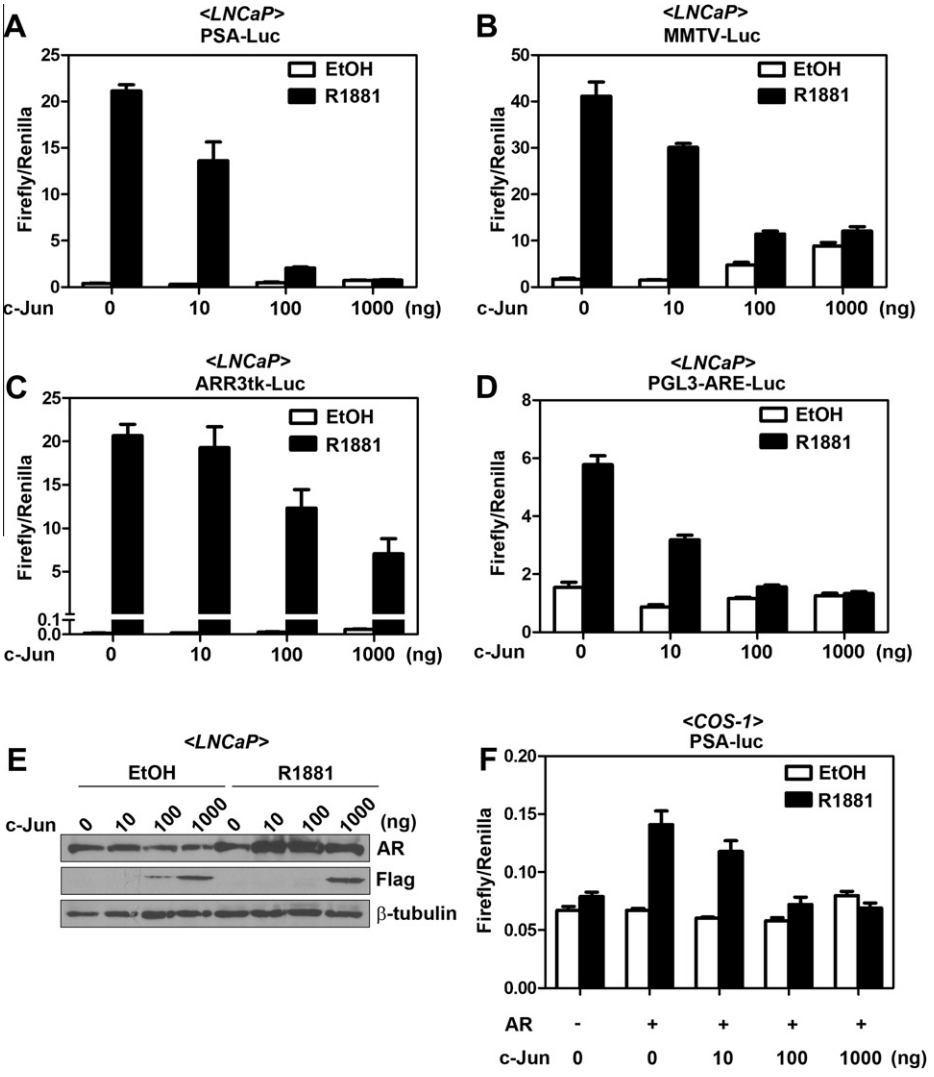

Fig. 1. c-Jun suppresses activities of multiple AR-responsive promoters. (A–D) Effects of c-Jun overexpression on the activities of various androgen-responsive promoters in LNCaP cells. LNCaP cells were transfected with 0.5 μg of luciferase reporter driven by *PSA* promoter (A), MMTV promoter (B), probasin promoter (C), or synthetic ARE (D), 100 ng of pRL-TK and increasing concentrations (0, 10, 100, and 1000 ng) of pFlag-c-Jun expression vector. After 24 h, cells were treated with ethanol (EtOH) or 10 nM R1881 for another 24 h. (E) Western blot analyzing the AR and Flag-c-Jun expression in luciferase lysates from (A). (F) Effect of c-Jun overexpression on AR-transactivated *PSA* promoter in COS-1 cells. COS-1 cells were co-transfected with 1 μg of empty vector or pHA-AR, 0.5 μg *PSA* promoter, 100 ng of pRL-TK and increasing concentrations (0, 10, 100, and 1000 ng) of pFlag-c-Jun expression vector. After 24 h, cells were treated with ethanol (vehicle control) or 10 nM R1881 for another 24 h. Error bars, S.E. (n = 3 for each experiment).

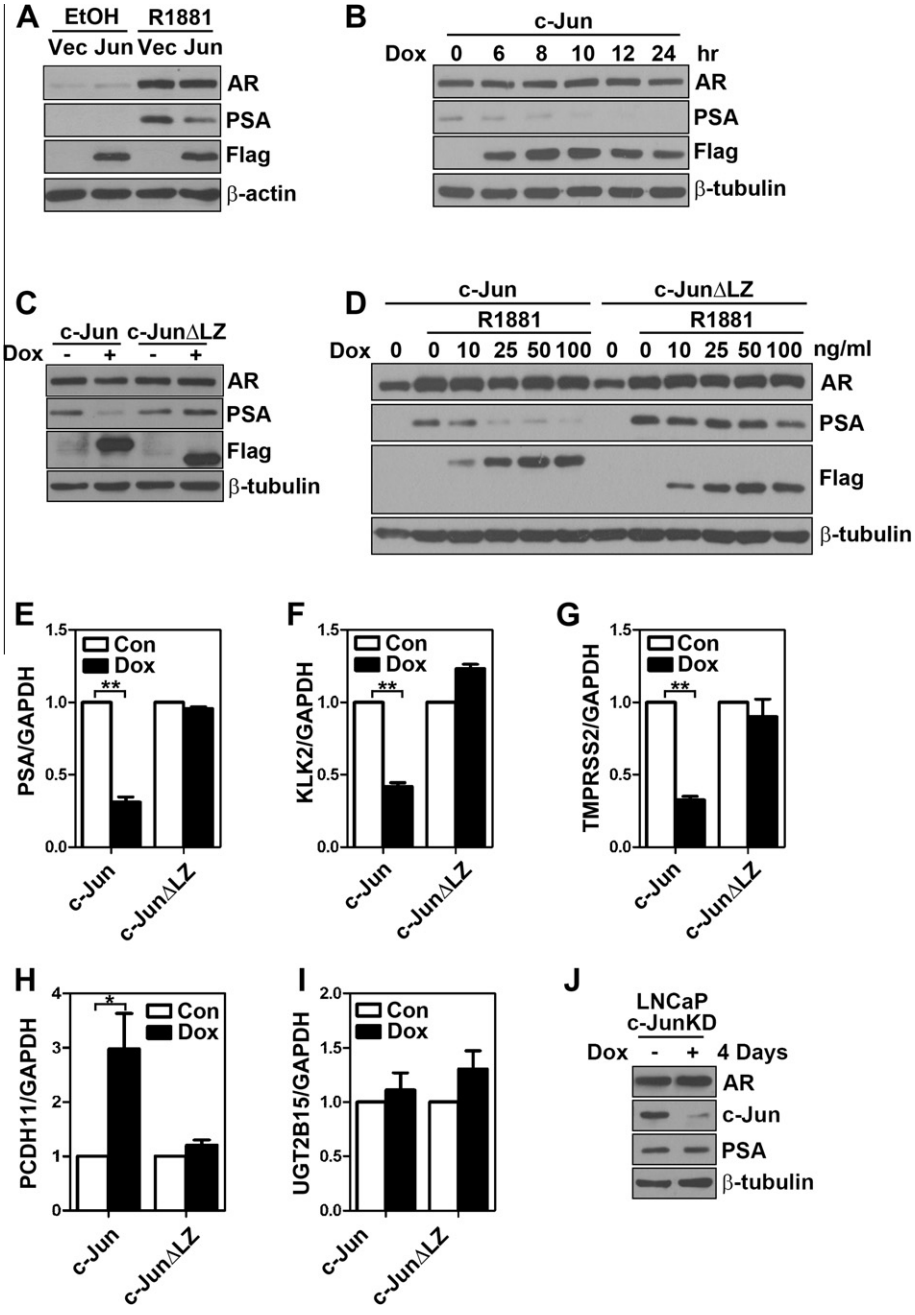

Fig. 2. c-Jun suppresses multiple AR target genes at endogenous level. (A) Effect of c-Jun overexpression on endogenous PSA level in LNCaP cells. LNCaP cells cultured in androgen-depleted medium for 24 h, followed by transient transfection with empty vector (Vec) or plasmids encoding Flag-c-Jun (Jun). After 24 h, cells were treated with 10 nM R1881 or the ethanol control (EtOH) for another 24 h before harvesting for immunoblotting analysis of AR, PSA and Flag-c-Jun (Flag). (B) Time course of c-Jun induction on endogenous PSA protein expression in LNCaP cells. LNCaP-rtTA-c-Jun cells were treated with 100 ng/ml doxycycline (Dox) for indicated periods of time. AR, PSA and Flag-c-Jun (Flag) were immunoblotted. (C) Effect of LZ deletion on c-Jun-inhibited PSA expression in LNCaP cells. LNCaP-rtTA-c-Jun or c-JunΔLZ cells were treated with 100 ng/ml Dox for 24 h. AR, PSA and Flag-c-Jun (Flag) were immunoblotted. (D) Wild-type but not LZ-deleted c-Jun efficiently suppresses androgen-induced PSA expression in LNCaP cells. LNCaP-rtTA-c-Jun or c-JunΔLZ cells were cultured in androgen-depleted medium for 24 h, followed by induction of wild-type or mutant c-Jun with indicated concentrations of Dox. Twenty-four hours post c-Jun induction, cells were treated with 10 nM R1881 for another 24 h. AR, PSA and Flag-c-Jun (Flag) were then immunoblotted. (E–I) Effect of c-Jun or c-JunΔLZ expression on steady state mRNA levels of *PSA*, *KLK2* ,*TMPRSS2*, *PCDH11* and *UGT2B15* in LNCaP cells. LNCaP-rtTA-c-Jun or c-JunΔLZ cells were treated with 100 ng/ml Dox for 24 h. Transcripts of *PSA* (E, $n = 4$), *KLK2* (F, $n = 3$), *TMPRSS2* (G, $n = 3$), *PCDH11* (H, $n = 3$), and *UGT2B15* (I, $n = 3$) were determined by qRT-PCR. The single and the double asterisk indicate the *P* value between control (Con) and Dox groups is less than 0.05 and 0.001, respectively (Student's *t*-test). (J) Effect of c-Jun knockdown on PSA expression in LNCaP cells. LNCaP-c-JunKD cells were treated with Dox (100 ng/ml) for 4 days followed by immunoblotting analysis of AR, PSA and c-Jun expression.

GAT TTT CAT GGA GAG G-3′; UGT2B15 R: 5′-GCT TTC CCC ATT GTC TCA AA-3′; GAPDH F: 5′-CTG ACT TCA ACA GCG ACA CC-3′; GAPDH R: 5′-CCC TGT TGC TGT AGC CAA AT-3′; AR F: 5′-GTG GAA GCT GCA AGG TCT TC-3′; AR R 5′-CGA AGA CGA CAA GAT GGA CA-3′. AR nascent RNA was measured by the following forward and reverse primers that recognize exon 1 and intron 1 of AR gene, respectively. AR nascent F: 5′-GGT GAG CAG AGT GCC CTA TC-3′; AR nascent R: 5′- GCG ACA TTT CTG GAA GGA AA-3′.

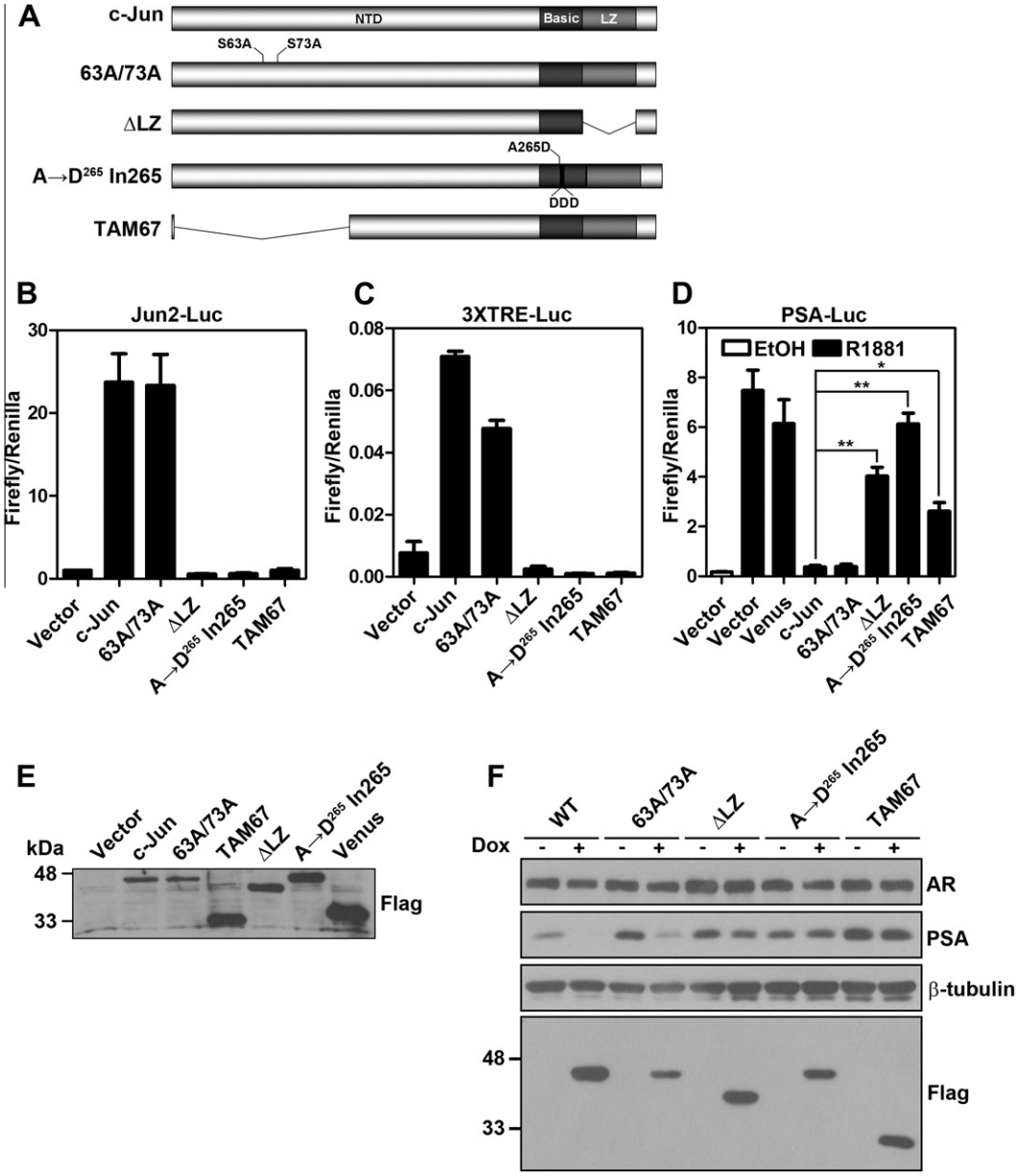

Fig. 3. The transcriptionally inactive c-Jun mutants fail to efficiently suppress AR activity. (A) Schematic view of various c-Jun mutants. NTD, N-terminal domain; LZ, leucine zipper, (B) and (C) Transcriptional activity of c-Jun mutants determined by luciferase reporters driven by the *jun2* response element or three tandem repeats of TRE. LNCaP cells were transfected with 0.5 μg of *jun2*-luc (B) or 3XTRE-luc (C), 100 ng of pRL-TK along with 1 μg of pCMV-Flag (vector) or indicated pFlag-c-Jun (wild type or mutants) for 24 h before assaying the luciferase activities. Error bars, S.E. ($n = 3$). (D) Effects of c-Jun mutants on AR activity in LNCaP cells were evaluated by *PSA* reporter utilizing the method as described in legend of Fig. 1. Error bars, S.E. ($n = 3$–5). The asterisk and the double asterisk indicate $P < 0.05$ and $P < 0.005$, respectively (Student's *t*-test). (E) Examination of the exogenous c-Jun expression in (D) by immunoblotting with a Flag antibody. (F) Effect of Flag-c-Jun mutants on endogenous PSA expression. LNCaP-rtTA-c-Jun cells (wild-type or mutants) were treated with 100 ng/ml Dox for 24 h, followed by immunoblotting for AR, PSA and Flag-c-Jun proteins expression. All Flag-c-Jun mutants (Flag) were detected with the anti-Flag antibody.

2.7. Cell counting analysis

LNCaP or C4-2 stable cell lines were seeded in regular medium at a density of 5×10^5 cells/10 cm dish. On the next day, designated day 0, cells from one dish were trypsinized for determining starting cell number by trypan blue exclusion assay (Strober, 2001). At the same time, cells in other dishes were treated with or without 100 ng/ml Dox. Cell number was counted on day 1, 3 and 5 followed by Dox treatment. Medium with indicated amounts of Dox was refreshed on day 1 and 3. Results are presented as mean ± S.E. from at least three independent experiments.

2.8. Cell cycle analysis

LNCaP- or C4-2-rtTA-c-Jun cells were treated with or without Dox for 3 days using same protocol as described in cell counting analysis. Cells were then trypsinized and fixed with ice-cold 70% ethanol. Followed by 30 min incubation at 4 °C, fixed cells were resuspended in PBS solution containing 100 μg/ml RNase A and 20 μg/ml propidium iodide. After 1 h incubation at room temperature, DNA contents of cells were measured by a CytomicsTM FC 500 (Beckman Coulter). Cell cycle was then analyzed using the Dean-Jett-Fox algorithm of FlowJo software.

2.9. BrdU incorporation assay

The bromodeoxyuridine (BrdU) incorporation assay was performed using BrdU and its antibody from BrdU Cell Proliferation Assay Kit (Calbiochem Cat#QIA58). LNCaP- or C4-2-rtTA-c-Jun cells (8.3×10^4 cells/well) were seeded onto coverslips in six-well plates overnight. Followed by treatment with or without Dox for 3 days, cells were labeled with BrdU for 4 h and then fixed by 70% ice-cold ethanol for 5 min. Cells were then incubated with 1.5 N HCl for 30 min at RT. After washing with PBS, cells were blocked by 5% non-fat milk and stained by anti-BrdU antibody. Cells were then incubated with anti-mouse IgG Texas Red-conjugated secondary antibody and 2.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 1 h. The BrdU incorporated cells were examined by a Nikon TE2000-U inverted fluorescence microscope. All images were taken at 200× magnification and percentage of BrdU positive cells were shown as mean ± S.E. quantified from at least nine randomly selected fields.

2.10. Statistical analysis

The data were expressed as mean ± S.E. Statistical analysis was performed by the unpaired two-tailed student's *t* test analyzed by GraphPad Prism 5 (La Jolla, CA).

3. Results

3.1. c-Jun inhibits activities of multiple AR-responsive promoters

We examined the role of c-Jun overexpression in the activation of several AR-regulated promoters in LNCaP prostate cancer cells that express endogenous AR. As shown in Fig. 1A–C, overexpressed c-Jun not only inhibited R1881-induced *PSA* promoter activity but also suppressed the activities of the mouse mammary tumor virus promoter (MMTV-Luc) and the probasin promoters (ARR3tk-luc) in a dose-dependent manner. To rule out the possibility that c-Jun inhibited the androgen-induced reporter gene activities by binding to AP-1 sites in the promoter regions, we examined the effect of c-Jun on luciferase reporter driven by tandem repeats of ARE. As shown in Fig. 1D, c-Jun also suppressed the R1881-induced ARE-Luc reporter (Fig. 1D), suggesting that c-Jun inhibits the AR responsive reporter without binding to the promoter regions. Overexpression of c-Jun did not suppress R1881-induced AR protein expression (Fig. 1E), suggesting that c-Jun could inhibit AR activity without affecting its protein level. Taken together, these results suggest that c-Jun exerts a global inhibitory effect on the AR activity in LNCaP cells.

Because the AR in LNCaP cells harbors a T877A mutation in LBD (Sobel and Sadar, 2005), we next sought to determine if c-Jun also inhibits function of wild-type AR. To this end, we performed similar *PSA* reporter assay in AR-negative COS-1 cells. Effects of c-Jun overexpression on *PSA* promoter activity were evaluated in COS-1 cells with or without co-transfection of plasmids encoding wild-type AR. As shown in Fig. 1F, reconstitution of AR expression is prerequisite for the induction of *PSA* promoter activity by R1881 in COS-1 cells. Hence, the system allows us to specifically examine the function of AR. Consistent with the results from LNCaP cells, overexpressed c-Jun also inhibited the R1881-activated *PSA* reporter gene in COS-1 cells (Fig. 1F), suggesting that AR inhibitory function of c-Jun is not cell type-specific or restrict to the T877A AR mutant.

3.2. Overexpression of c-Jun suppresses multiple AR-regulated gene expression at endogenous level

To confirm these observations in a more physiologically relevant context, we examined the effect of c-Jun on androgen-induced PSA protein expression. Consistent with the observation in the promoter activity assay, transient expression of c-Jun suppressed R1881-induced PSA protein expression (Fig. 2A). To gain more insight into the effects of c-Jun on AR function, we utilized lentivirus to generate an inducible system in which c-Jun expression is controlled in a doxycycline (Dox)-dependent manner in LNCaP cells. Cells with inducible c-JunΔLZ were generated as a negative control based on a previous report that LZ region is responsible for its interaction with AR (Sato et al., 1997). The purpose of using Dox-inducible system was to prevent artifacts during clonal selection of c-Jun overexpressing cells. Treatment of cells with Dox (100 ng/ml) for 2 days induced the expression of exogenous Flag-c-Jun in ~70% of cells (data now shown). We first examined the time course of c-Jun induction on endogenous PSA protein levels. As shown in Fig. 2B, the endogenous PSA protein expression was reduced within 6 h upon c-Jun induction. The down-regulation of PSA sustained at least to 24 h, at which time point no obvious change in AR expression was observed. Induction of c-Jun, but not c-JunΔLZ, down-regulated both steady state and androgen-induced PSA protein expression (Fig. 2C and D). Furthermore, induction of c-Jun, but not c-JunΔLZ, also significantly down-regulated steady state mRNA levels of *PSA* and two other androgen-regulated genes, *KLK2* and transmembrane protease, serine 2 (*TMPRSS2*) (Fig. 2E–G), suggesting that c-Jun globally inhibits AR transactivation function. Because AR was also shown to function as a transcription repressor, we then examined the effects of c-Jun on two AR-repressed genes, *PCDH11* (Yang et al., 2005) and *UGT2B15* (Bao et al., 2008). Although c-Jun overexpression caused a ~2-fold increase of *PCDH11* transcripts (Fig. 2H), it did not affect the expression of *UGT2B15* (Fig. 2I), suggesting that c-Jun alleviates AR transrepression function in a gene context-dependent manner. Taken together, our results strongly suggest that c-Jun is a potent inhibitor for the AR transactivation function. To determine if endogenous c-Jun suppresses AR activity, we generated an LNCaP stable cell line in which c-Jun expression can be inducibly knocked down by Dox. Interestingly, no significant change in PSA protein level was observed in c-Jun knockdown cells (Fig. 2J), suggesting that basal level of c-Jun in LNCaP cells is not sufficient to inhibit AR function. In fact, the steady state c-Jun level in LNCaP cells is relatively low compared with PC3 or DU145 cells, two other human prostate cancer cell lines with little AR expression (Ouyang et al., 2008; Sun et al., 1999).

3.3. The AR inhibitory function correlates to the transactivational activity of c-Jun

It was reported that c-Jun may inhibit the AR activity by its physical interaction with AR via its leucine zipper region (Sato et al., 1997). This conclusion was largely based on the observation that deletion of the zipper region abrogated the AR inhibitory function. Since transcriptional function of AP-1 proteins is dependent on dimerization, c-Jun mutant without LZ region is also transcriptionally inactive. To further determine whether the inhibition of the AR activity by c-Jun is mediated by its AP-1 activity or by its physical interaction with AR, we tested the AR-inhibitory function of several c-Jun mutants with impaired transactivation function. The mutants used include: c-Jun63A/73A, in which both serines 63 and 73 were replaced with non-phosphorylatable alanines; c-JunΔLZ, in which LZ region was deleted; c-Jun A→D^{265} In265 whose DNA binding activity is abrogated by introduction of aspartic acids in the DBD (Brown et al., 1996); c-JunΔ3-122 (TAM67), in which the N-terminal transactivation domain was deleted (Fig. 3A) (Brown et al., 1996). It is important to note that TAM67 and the DNA binding-deficient mutant retain the ability to dimerize. The transcriptional activity of these mutants was first evaluated using two different c-Jun responsive promoters: luciferase reporters

driven by a *jun2* response element or by three tandem repeats of TRE. We confirmed that c-JunΔLZ, c-Jun A→D^{265} In265 and TAM67 are transcriptionally inactive (Fig. 3B and C). Surprisingly, the 63A/73A mutation only partially reduced (~30% reduction) the transactivation potential of c-Jun on 3XTRE promoter activity. Importantly, consistent with our previous observation (Liu et al., 2006a), the non-phosphorylatable mutant activated the *jun2*-promoter activity as well as wild-type c-Jun. These results suggest that the effect of the double alanine mutation on c-Jun transcriptional activity is promoter context-dependent and that this mutation does not always affect the transactivation potential of c-Jun. We then examined the effect of these mutants on R1881-induced *PSA* promoter activity. A Venus fluorescent protein was used as a negative control for the effect of protein overexpression on *PSA* promoter activity. Overexpression of Venus slightly reduced androgen-induced *PSA* promoter activity, which may result from nonspecific competition for transcription and translation machinery (Fig 3D). The c-Jun63A/73A mutant inhibited AR activity as well as wild-type one (Figs. 3D and S1), suggesting that the phosphorylation event at these two serine residues is dispensable for the AR inhibitory function. Consistent with the finding of Sato et al. (1997), deletion of the LZ region significantly alleviated the c-Jun inhibitory activity. Remarkably, the AR inhibitory function of c-Jun was completely lost in DNA binding-deficient mutant (c-Jun A→D^{265} In265) and was significantly attenuated in the transactivation domain-deleted mutant (TAM67). Immunoblotting analysis verified that the lack of the AR inhibitory role was not due to lower expression of these mutants. In fact, the expression level of these transcriptionally inactive Jun proteins was even slightly higher than wild-type c-Jun (Fig 3E). To further confirm the effects of these mutants on AR function in a more physiologically relevant context, we examined PSA protein levels in LNCaP stable transfectants expressing various inducible c-Jun mutants. As shown in Fig. 3F, endogenous PSA protein of LNCaP cells was significantly down-regulated by induction of c-Jun or c-Jun63A/73A, but not c-JunΔLZ, c-Jun A→D^{265} In265 or TAM67. Taken together, these results suggest that transcriptional activity of c-Jun is critical for its AR inhibitory role.

3.4. Long-term c-Jun overexpression leads to down-regulation of AR protein

We next determined the long-term effect of c-Jun expression on AR signaling. Interestingly, AR protein was significantly decreased 3 days after c-Jun induction (Fig. 4A). To determine the mechanism underlying the down-regulation of AR protein, we evaluated if c-Jun regulates AR expression at the levels of transcription or protein stability in LNCaP cells. We found that *AR* mRNA level was significantly down-regulated (~37% reduction) at 24 h post-c-Jun induction (Fig. 4B). A significant down-regulation of nascent *AR* RNA transcript was also observed in c-Jun-overexpressing cells (Fig. 4C), suggesting that c-Jun reduces *AR* mRNA level by suppressing *AR* transcription. We then determine AR protein stability by treating cells with cycloheximide, a protein synthesis inhibitor. The cycloheximide treatment (for up to 9 h) did not significantly alter endogenous AR protein level in cells with c-Jun induction (Fig. 4D), suggesting that AR protein remained stable under the condition of c-Jun overexpression. Taken together, our results suggest that long-term c-Jun induction diminishes AR protein level by inhibiting *AR* transcription.

3.5. Overexpression of c-Jun inhibits proliferation of LNCaP cells

It is well known that AR signaling axis is required for growth of androgen-dependent prostate cancer cells. Our result that c-Jun inhibits AR function prompted us to test if induction of c-Jun

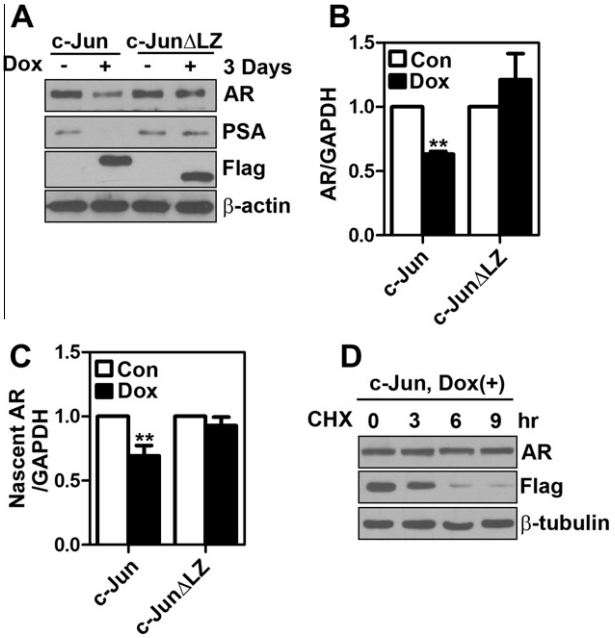

Fig. 4. Long-term c-Jun induction down-regulates AR expression at transcription level. (A) LNCaP-rtTA-c-Jun or c-JunΔLZ cells were treated with 100 ng/ml doxycycline (Dox) for 72 h, followed by immunoblotting analysis of AR, PSA and Flag-c-Jun expression. (B and C) LNCaP-rtTA-c-Jun or c-JunΔLZ cells were treated with Dox (100 ng/ml) for 24 h. Transcripts of *AR* mRNA (B, $n = 4$) and nascent *AR* RNA (C, $n = 4$) were determined by qRT-PCR. The double asterisk indicates the *P* value between control (Con) and Dox groups is less than 0.01 (Student's *t*-test). (D) LNCaP-rtTA-c-Jun cells were treated with Dox (100 ng/ml) for 24 h followed by cycloheximide (10 μg/ml) treatment for indicated periods of time. AR and Flag-c-Jun were immunoblotted.

affects proliferation of LNCaP cells. LNCaP-rtTA-c-Jun, c-JunΔLZ or c-Jun A→D^{265} In265 cells were treated with or without Dox (100 ng/ml) and cell numbers were counted on day 0, 1, 3 and 5. As shown in Fig. 5A, treatment of LNCaP-rtTA-c-Jun with 100 ng/ml Dox for 5 days significantly reduced the cell number from 43.9 ± 8.2 to $15.7 \pm 2 \times 10^5$. In contrast, neither c-JunΔLZ nor c-Jun A→D^{265} In265 induction significantly reduced cell number. Flow cytometry analysis showed that c-Jun induction for 3 days suppressed cell cycle progression, as evidenced by increased G1 population and reduced S and G2/M populations (Fig. 5B). Moreover, c-Jun induction also suppressed the BrdU incorporation rate (Fig 5C), confirming the suppressive role of c-Jun in LNCaP cell proliferation. On the other hand, we did not observe poly (ADP-ribose) polymerase (PARP) cleavage, an apoptosis marker, in LNCaP cells that overexpressed c-Jun (Fig. S2), suggesting that the reduced cell number of cells is primarily due to the inhibition of cell proliferation rather than the induction of apoptosis. Taken together, c-Jun not only inhibits AR activity but also cell proliferation of androgen-dependent prostate cancer cells.

3.6. c-Jun inhibits AR function in castration-resistant prostate cancer cells

Several previous studies showed that a subset of castration-resistant prostate cancer patients also showed c-Jun down-regulation (Edwards et al., 2004; Tamura et al., 2007), though its clinical significance remains unclear. To determine whether c-Jun overexpression may have a differential effect on hormone naïve prostate cancer cells and CRPC cells, we next examined the role of c-Jun in CRPC cells. For the purpose of comparison, we chose the C4-2 cell line, which is widely used as a CRPC line and is derived from LNCaP cells (Wu et al., 1994). We found transfection of c-Jun dose-dependently inhibited R1881-indeuced *PSA* promoter activity

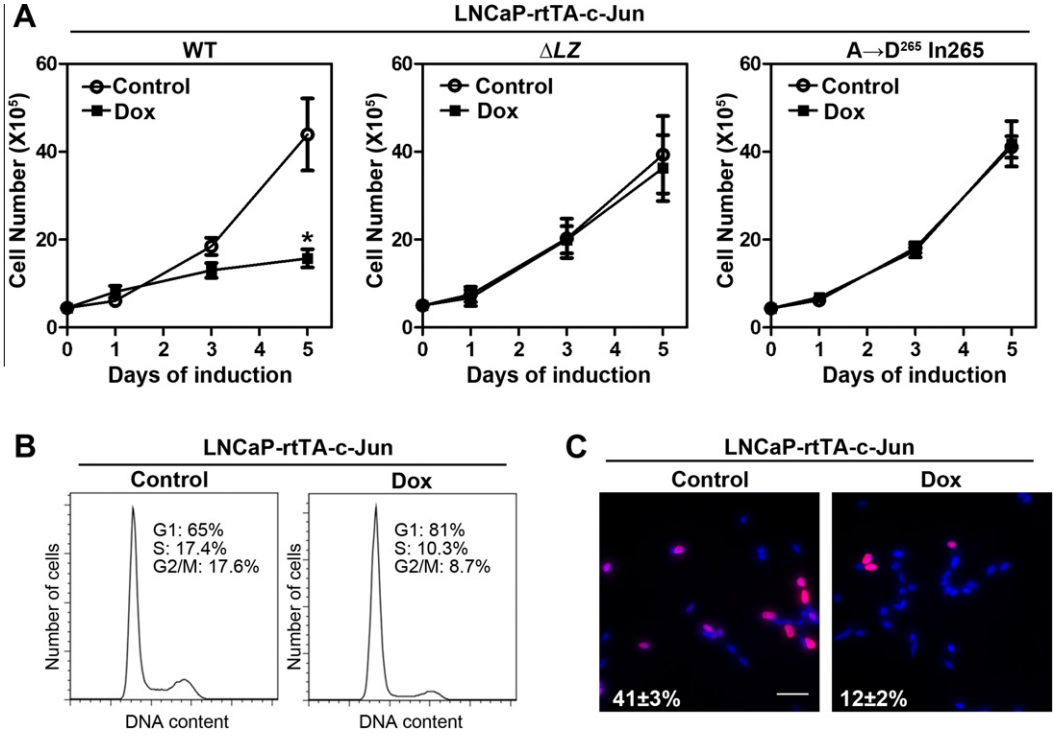

Fig. 5. Overexpression of c-Jun inhibits proliferation of LNCaP cells. (A) Effect of c-Jun overexpression on cell number of LNCaP cells. LNCaP-rtTA-c-Jun cells (wild-type or mutants) were treated with or without 100 ng/ml of doxycycline (Dox) for 5 days. Cell numbers were counted on day 0, 1, 3, and 5. The asterisk indicates a significant difference ($P < 0.05$) when compared with the control group (Student's *t*-test). Error bars, S.E. ($n = 3$). (B) Effect of c-Jun overexpression on cell cycle progression of LNCaP cells. LNCaP-rtTA-c-Jun cells were treated with or without Dox for 3 days, followed by flow cytometry analysis of cell cycle progression. (C) Effect of c-Jun overexpression on DNA synthesis of LNCaP cells. LNCaP-rtTA-c-Jun cells were treated with or without Dox for 3 days and followed by BrdU incorporation assay. Shown are representative merged images of BrdU labeling (red) and DAPI staining (blue). Bar, 100 μm. The number indicates the percentage of BrdU incorporated cells ± S.E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in C4-2 cells as well (Fig 6A). We similarly generated C4-2 stable cell lines with inducible expression of c-Jun proteins and found that like in LNCaP cells, c-Jun, but not c-JunΔLZ or c-Jun A→D^{265} In265, suppressed endogenous PSA expression (Fig. 6B). Interestingly, overexpression of c-Jun, but not c-JunΔLZ or c-Jun A→D^{265} In265, for 5 days also reduced cell number of C4-2 cells (Fig. 6C). Overexpression of c-Jun inhibited cell cycle progression (Fig. 6D) and BrdU incorporation (Fig. 6E) in C4-2 cells, albeit to a lesser extent compared to LNCaP cells. The decreased anti-proliferative effect in C4-2 cells as opposed to LNCaP cells was likely due to lower induction of Flag-c-Jun expression in C4-2 cells (data not shown). Our observations are consistent with previous reports that AR signaling remains important for proliferation of CRPC cells (Chen et al., 2004; Snoek et al., 2009; Yuan et al., 2006; Zegarra-Moro et al., 2002). Taken together, these results suggest that c-Jun is capable of suppressing AR function, through a mechanism that depends on the transcriptional activity of c-Jun, in prostate cancer cells that acquired castration resistance.

4. Discussion

The interplay between AP-1 and steroid hormone receptors has been extensively studied (Herrlich, 2001; Karin and Chang, 2001; Kushner et al., 2000; Pfahl, 1993). With regard to the impact of c-Jun on AR signaling, both stimulatory (Bubulya et al., 2001, 2000, 1996; Chen et al., 2006; Shemshedini et al., 1991; Wise et al., 1998) and inhibitory (Chung et al., 2001; Lobaccaro et al., 1999; Murtha et al., 1997; Sato et al., 1997; Yuan et al., 2010) effects were reported and these observations suggested that c-Jun may act as a coactivator or corepressor of AR by physically interacting with AR. In the present study, we provide evidence here supporting that c-Jun is a potent inhibitor for AR function and the transcriptional activity of c-Jun, rather than the AR/c-Jun interaction, plays a major role for this inhibition. First, c-Jun dose-dependently inhibited activity of four AR responsive luciferase reporters. Second, c-Jun overexpression suppressed steady state and androgen-induced PSA protein expression in LNCaP cells. Third, c-Jun down-regulated mRNA levels of multiple AR target genes, including *PSA*, *KLK2* and *TMPRSS2*. Forth, the inhibitory effect of c-Jun on *PSA* promoter activity and protein expression was significantly alleviated in transactivation-deficient mutants, including the transactivation domain-deleted (TAM67), dimerization- (c-JunΔLZ) and DNA binding-deficient (c-Jun A→D^{265} In265) mutants.

The impact of c-Jun on AR activity has been a controversial issue. Although c-Jun was reported to potentiate AR activity (Bubulya et al., 2001, 2000, 1996; Chen et al., 2006; Shemshedini et al., 1991; Wise et al., 1998), the opposite effects were observed by others (Chung et al., 2001; Lobaccaro et al., 1999; Murtha et al., 1997; Sato et al., 1997; Yuan et al., 2010). Interestingly, the use of AR ligands is the major discrepancy, with DHT and R1881 been utilized by groups reported that c-Jun positively and negatively regulates AR activity, respectively. Because DHT, but not R1881, is known to be rapidly converted to other inactive metabolites in many cell lines (Bjelfman et al., 1997; Martini, 1982; Negri-Cesi and Motta, 1994), DHT does not stimulate AR activity as effectively as R1881. Indeed, DHT only weakly stimulated MMTV promoter activity in reports showing that c-Jun enhanced AR activity (Bubulya et al., 1996; Shemshedini et al., 1991; Wise et al., 1998). Interestingly, we found that c-Jun stimulated MMTV promoter activity in the absence of androgen (Fig. 1B), presumably due to the binding to the putative AP-1 sites reported (Vacca et al., 1989). Thus, a

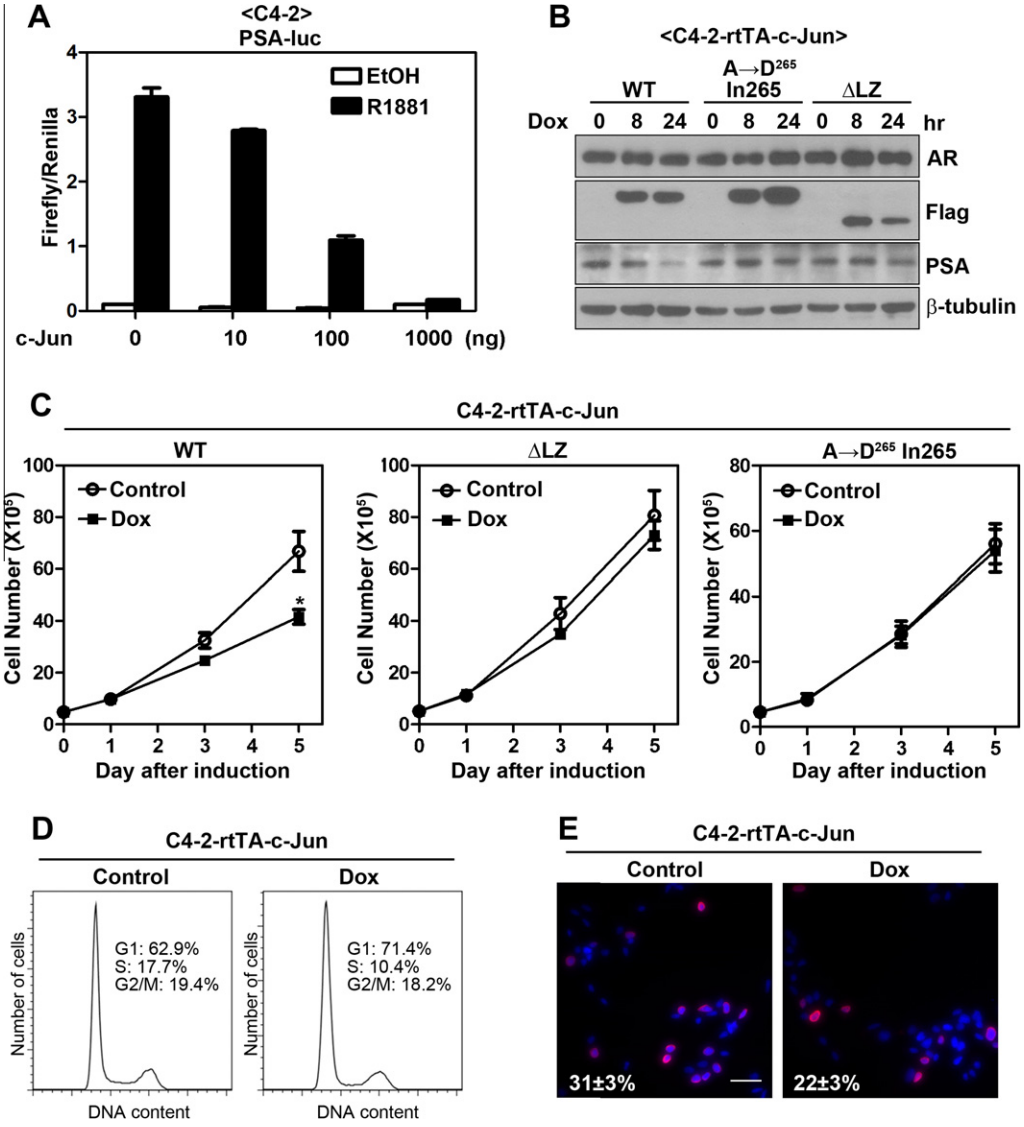

Fig. 6. c-Jun suppresses AR function in castration-resistant prostate cancer cells. (A) Effect of c-Jun overexpression on R1881-induced *PSA* promoter activity in C4-2 cells. *PSA* reporter assay was performed in C4-2 cells using method as described in Fig. 1A. Error bars, S.E. ($n = 3$). (B) Induction of c-Jun inhibits endogenous PSA expression in C4-2 cells. C4-2-rtTA-c-Jun cells (wild-type or mutants) were treated with 100 ng/ml of doxycycline (Dox) for indicated periods of time. AR, PSA and Flag-c-Jun (Flag) proteins were immunoblotted. (C) Overexpression of c-Jun reduces cell number of C4-2 cells. Effects of c-Jun (wild-type or mutants) induction on cell number of C4-2 cells were determined using the method as describe in Fig. 5A. The asterisk indicates a significant difference ($P < 0.05$) when compared with the control group (student's *t*-test). Error bars, S.E. ($n = 4$ for c-Jun or c-JunΔLZ; $n = 3$ for c-Jun A→D^{265} In265). (D) Effect of c-Jun overexpression on cell cycle progression of C4-2 cells. C4-2-rtTA-c-Jun cells were treated with or without Dox for 3 days, followed by flow cytometry analysis of cell cycle progression. (E) Effect of c-Jun overexpression on DNA synthesis of C4-2 cells. C4-2-rtTA-c-Jun cells were treated with or without Dox for 3 days and followed by BrdU incorporation assay. Shown are representative merged images of BrdU labeling (red) DAPI staining (blue). Bar, 100 μm. The number indicates the percentage of BrdU incorporated cells ± S.E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

more careful examination is needed when using MMTV promoter to determine the impact of c-Jun on AR function. On the other hand, it was reported that LNCaP cells stably overexpressing c-Jun exhibited higher AR activity (Chen et al., 2006). Our results that c-Jun induction suppressed growth of LNCaP cells raised the concern that generation of stable cell line with constitutive c-Jun overexprssion could likely confer a selection pressure which favors the growth of clones that are less dependent on AR signaling for proliferation. Hence, we believe our strategy of utilizing the inducible expression system allowed us to investigate the impact of c-Jun on AR function more accurately.

It was also proposed that AP-1 inhibits AR activity by competing with limited amount of CREB binding protein (CBP), which serves as a coactivator for both AR and c-Jun (Fronsdal et al., 1998). The amino-terminal region, especially the Ser-63/73, of c-Jun is required for the interaction with CBP (Bannister et al., 1995). Because AR activity was efficiently inhibited by c-Jun63A/73A but not by the bZIP mutants whose N-terminal domains remain intact, the repression is less likely due to the competition with limited amount of CBP. Instead, our finding that AR inhibition was significantly attenuated in c-Jun mutants that lose the transcriptional activity suggests that c-Jun may indirectly inhibit AR function via activating a downstream target gene. Since cyclin D1 was shown to be a c-Jun target gene (Albanese et al., 1995; Bakiri et al., 2000; Herber et al., 1994) and an AR corepressor (Comstock et al., 2011; Petre et al., 2002; Petre-Draviam et al., 2003), we considered the possibility that cyclin D1 may mediate the inhibitory effect. However, we did not observe a significant increase in cyclin D1 expression at both mRNA and protein levels in LNCaP cells with c-Jun induction (data not shown). Thus, c-Jun may inhibit AR

through transactivating downstream target genes other than cyclin D1. A microarray analysis of differentially expressed genes in LNCaP cells with or without c-Jun induction may help identify the critical target genes that are responsible for the AR inhibition.

One interesting finding is that c-Jun also down-regulated the expression of AR at the protein level after three days induction (Fig. 4A), whereas induction of c-Jun for one day only affects the expression of *AR* at the transcript level (Fig. 2B and 4B). Because of high stability of AR protein in LNCaP cells cultured in regular medium (Fig. 4D), it may take longer time (more than 24 h) to see any significant change in protein level. Because PSA expression is reduced as early as 6 h followed by c-Jun induction when AR protein remains unchanged (Fig. 2B), the down-regulation of AR protein is not essential for the inhibition of AR function. Nevertheless, sustained elevation of c-Jun expression could further suppress AR signaling by inhibiting AR expression. Thus, the elevated c-Jun level may inhibit AR signaling axis via a dual mechanisms; with a suppression of AR activity first and a down-regulation of AR protein level later. In addition to functioning as a transcriptional activator, c-Jun was also reported to suppress gene expression by binding to the promoter region (Ivanov et al., 2001; Schreiber et al., 1999). Further experiments are needed to clarify if c-Jun inhibits *AR* transcription directly via binding to the AP-1 sites identified in human *AR* promoter (Mizokami et al., 1994). Alternatively, c-Jun *per se* does not inhibit *AR* transcription. Instead, c-Jun target genes may inhibit *AR* transcription.

In conclusion, our results suggest that the transcriptional activity of c-Jun is required for its efficient inhibition of AR function. Given that both the AR activity and the proliferation of hormone naïve prostate cancer cells and CRPC cells are suppressed by c-Jun overexpression, identification of the critical c-Jun downstream target genes will provide a novel therapeutic strategy aimed at treating a subset of prostate cancer in which c-Jun down-regulation may underly the progression of the disease.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.03.004.

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Education / Degrees Awarded:

9/1979-**7**/1984: Bachelor in Medical Science (Equivalent to ***M.D.***)
Faculty of Medicine, Bengbu Medical College, Bengbu, China

9/1984-7/1987: ***M.S.*** (Cancer Immunology)
Department of Microbiology and Immunology, Faculty of Medicine,
Tongji Medical University, Wuhan, China

4/1994-3/1997: ***Ph. D***. (Molecular biology)
Department of Physiology II, Kobe University School of Medicine, Japan

Teaching Experience:

5/1988-6/1987:	Microbiology and Immunology labs (medical students)
7/1987-8/1991:	Epidemiology lectures and labs in the Department of Epidemiology, School of Public Health, Tongji Medical University, Wuhan
4/1994-8/2000:	Physiology and Molecular Biology labs (medical students) in the Department of Physiology II, Kobe University
8/2003-present:	Biochemistry and Pathophysiology (Pharmacy students) Other graduate courses in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University

Research/Working Experience:

9/1984-7/1987: ***Graduate Student (M.S.)*** in the Department of Microbiology & Immunology, Tongji Medical University, Wuhan, China.
Study of anti-tumor mechanisms of a new Chinese herb medicine in cell culture and animal models.

7/1987-9/1991*:* ***Lecturer*** in the Department of Epidemiology, School of Public Health, Tongji Medical University, Wuhan, China.
(1). Study on the mutagenicity of trichloromethane

(2). Epidemiological investigation of drinking water and cancer incidence in Wuhan, China.

9/1991-3/1994: ***Guest Research Associate*** in the Department of Molecular Oncology, Kyoto University School of Medicine, Kyoto, Japan.
(1). Spontaneous and induced acquisition of tumorigenicity in nude mice by lymphoblastoid cell line derived from patients with xeroderma pigmentosum group A.
(2). Subtractive isolation of genes contributing to the acquisition of tumorigenicity by lymphoblastoid cell line derived from xeroderma pigmentosum group A patient.

4/1994-3/1997: ***Graduate Student (Ph.D.)*** in the Department of Physiology II, Kobe University School of Medicine, Kobe, Japan
(1). Regulation of Raf-1 kinase activity by Ha-Ras and Rap1A.
(2). Activation mechanism of Ras effectors.

4/1997-8/2000: ***Assistant Professor*** in the Department of Physiology II, Kobe University School of Medicine, Kobe, Japan.
(1). Regulation of Raf kinase activity by Ha-Ras and Rap1A.
(2). Identification and characterization of novel Ras effectors and regulators.
(3). Activation mechanism of Ras effectors.

9/2000-6/2003: ***Research Investigator/Specialist*** in the Department of Biological Chemistry and Howard Hughes Medical Institute, University of Michigan School of Medicine.
(1). Establishment of bimolecular fluorescence complementation (BiFC) and multicolor bimolecular fluorescence complementation (MuFC) assays for the study of protein-protein interaction in living cells.
(2). Functional analysis of cross-family transcription factor interactions among bZIP, Rel, Smad and Myc/Max families.
(3). BiFC analysis of protein-protein interactions in *C. elegans.*

7/2003-2009: ***Assistant Professor*** in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University School of Pharmacy.
(1) Development and improvement of BiFC-based technologies
(2) AP-1 in *C. elegans* development
(3) AP-1 in prostate cancer development and therapeutic responses

8/2009- ***Associate Professor*** (tenured) in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University School of Pharmacy.
(1) Development and improvement of BiFC-based technologies
(2) Mechanisms and targeting of therapy-resistant prostate cancer
(3) Development of novel radiosensitizers and chemosensitizers of lung cancer
(4) Development of high throughput screening for discovery of inhibitors of protein-protein interactions

Award:

09/91-09/92:	Fellowship of JSPS Source: **J**apan **S**ociety for the **P**romotion of **S**cience
09/92-09/93:	Kyoto University Alumni Fellowship Source: Kyoto University
04/94-03/97	Senshukai Scholarship (Ph.D. student) Source: Kobe Senshukai Scholarship Foundation
04/98-03/99	President Young Investigator Award Source: Kobe University
04/98-03/99	Young Investigator Award Source: JSPS
04/99-03/01	Young Investigator Award Source: Hyogo Prefecture Science and Technology Association

Current and Past Grant Support:

Past Grant Support

04/98-03/99	Regulation of Rap1A activity by phosphorylation Source: Kobe University
04/98-03/99	Effect of phosporylation on the regulation of Rap1A activity Source: Japan Society for the Promotion of Science
04/00-03/01	Activation mechanism of phospholipase C (PLC-ε) by Ras Source: Hyogo Prefecture Science and Technology Association
04/00-03/01	Regulation of a novel phospholipase C (PLC-ε) by Ras Source: Japan Society for the Promotion of Science
08/04-07/08	Visualization of temporal and spatial interaction patterns of bZIP proteins in living *C. elegans* Source: National Science Foundation
07/06-06/08	Regulation of *c-jun* transcription by ATF2 in cardiomyocyte in response to stress Source: American Heart Association
03/08-02/09	Mass spectrometric identification of phospho-CREB in prostate cancer cells Source: Purdue University Center for Cancer Research
06/08-05/12	Interplay of ATF2 and pCREB in radiation-induced neuroendocrine differentiation in prostate cancer cells Source: Department of Defense (PCRP)
01/09-12/11	Targeting of prostate cancer transdifferentiation and proliferation via a novel DNA nanotube-based nucleic acid delivery Source: Lilly Seed Grant
01/09-12/11	Ionizing radiation induces neuroendocrine differentiation in nude mice prostate cancer xenograft models: Implication in disease progression Source: Purdue University Center for Cancer Research

06/10-05/12	Chromogranin A, a novel biomarker to monitor radiation-induced neuroendocrine differentiation in prostate cancer patients Source: The Indiana Clinical and Translational Science Institute (CTSI)-Purdue Project Development Program
06/10-12/11	Generation of cytoplasmic-localized ATF2 transgenic mice for prostate cancer research Source: Purdue University Center for Cancer Research

Current Grant Support

01/12-12/13	Improvement of BiFC technology and its application in the TLR signal transduction pathway (International collaborative project) Source: Natural Science Foundation of China
08/12-07/15	Targeting PRMT5 as a novel radiosensitization approach for primary and recurrent prostate cancer radiotherapy Source: DOD (Prostate Cancer Research Program)
08/13-07/16	Targeting neuroendocrine differentiation for prostate cancer radiotherapy Source: DOD (Prostate Cancer Research Program)
04/12-03/14	RO1: D2 receptor-induced sensitization of adenylate cyclase Source: NIH (Co-PI with Val Watts)
04/13-03/15	R21: Identification of Ac5 sensitization interactome using BiFC Source: NIH (Multi-PI with Val Watts)

Professional Services:

Memberships

2001-	American Association for Cancer Research
2001-	American Society for Biochemistry and Molecular Biology
2003-	American Society of Cell Biology
2004-	Genetics Society of America
2009-	Society for Basic Urological Research
2010-	American Urological Association

Reviewer for Grant Applications

2004	Reviewer of MAES (The Maryland Agricultural Experiment Station at the University of Maryland)
2005	*Ad hoc* reviewer for NSF Advisory Panel for Molecular and Cell Biology
2006-2008	American Heart Association
2007-present	Qatar National Research Fund (QNRF)
2008-present	Pennsylvania Department of Health (PADOH)
2008-present	UK Cancer Research, UK Diabetes
2009-present	Welcome Trust
2010-present	Department of Defense, Prostate Cancer Research Program (Immunology Panel, Endocrine Panel)

Reviewer for Professional Journals

Combinatory Chemistry and HTS, Zebrafish, Journal of Biological Chemistry, Molecular and Cellular Biology, Nature Biotechnology Nature Methods, Molecular Cell, Molecular Biology of the Cell, PNAS, BMC Biotechnology, BMC Biology, Biotechniques, Biochemistry, ACS Chemical Biology, Chemistry & Biology, Journal of Innovative Optical Health Sciences, TIBS, TIBT, Current Cancer Drug Targets, Journal of Cell Science

Editorial Board Member:

2007- Perspective in Medicinal Chemistry
2011- American Journal of Cancer Research

Members/Organizers of Meetings

Organizer of Bimolecular Fluorescence Complementation Workshop (Purdue University), 2010
Chair of Optical Molecular Imaging, 2008 PIBM
Chair of Imaging Technology Symposium, 2008 4th Modern Drug Discovery and Development Summit
Member of 2009 PIBM Program Committee
Organizer of Tristate Worm Meeting at Purdue (2005)

Invited Seminars/Conference Presentations:

05/24/13 Place: Hefei Chinese Academy of Sciences Cancer Hospital
Title: Impact of neuroendocrine differentiation in prostate cancer radiotherapy

05/20/13 Place: Huazhong University of Science and Technology Union Hospital Cancer Institute
Title: Radiation-induced neuroendocrine differentiation in prostate cancer: From bench to bedside

05/17/13 Place: Jinan University School of Medicine
Title: Neuroendocrine differentiation (NED) in prostate cancer cells: From basic science to clinical practice

05/14/13 Place: Northwestern Agriculture and Forestry University (NWAFU): 2013 Purdue-NWAFU Center Symposium
Title: Bimolecular fluorescence complementation (BiFC): Current Status and Future Perspectives

04/17/13	Place: 2013 Drug Discovery Chemistry in San Diego: Sixth Annual Protein-Protein Interactions (Targeting PPI for Therapeutic Interventions) Title: Bimolecular fluorescence complementation (BiFC) as a novel imaging-based screening for inhibitors of protein-protein interactions. Moderator of Breakout Discussion: Image-based HTS for PPIs
02/05/13	Place: Tongji Hospital, HUST Title: Neuroendocrine differentiation (NED): A therapeutic challenge in prostate cancer management
10/25/12	Place: Wright State University Department of Biochemistry and Molecular Biology Title: Bimolecular fluorescence complementation (BiFC): An imaging tool for visualization of molecular events
06/06/12	Place: Jiangshu University School of Medical Technology and Laboratory Medicine Title 1: Mechanisms and targeting of radiation-induced neuroendocrine differentiation Title 2: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
06/4/12	Place: Chinese Academy of Sciences (Hefei) Title: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
05/31/12	Place: Tongling Traditional Chinese Medicine Hospital Title: Recent advances in prostate cancer diagnosis and treatment
05/18/12	Place: Shanghai Center for Plant Stress Biology of Chinese Academy of Sciences Title: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
04/25/12	Place: University of Western Ontario Title: Radiotherapy-induced neuroendocrine differentiation: Implications in prostate cancer progression and treatment
03/13/12	Place: Mayo Clinic Title: Mechanisms and targeting of therapy-induced neuroendocrine differentiation for prostate cancer treatment
07/11/11	Place: Jinan University Medical School

Title: Bimolecular fluorescence complementation: An emerging technology for biological research

07/10/11 Place: Sun-Yat-sun University Medical School
Title: Mechanisms and targeting of therapy-resistant prostate cancer

02//09/11 Place: Tulane University Medical School
Title: Mechanisms and targeting of therapy-resistant prostate cancer

01/17/11 Place: Penn State College of Medicine
Title: Bimolecular fluorescence complementation (BiFC): Current Challenges and Future Developments

12/07/10 Place: Purdue University BiFC Workshop
Title: Bimolecular fluorescence complementation: principle, experimental design and data analysis
Organizer and Speaker: BiFC Workshop

11/18/10 Place: UT Austin College of Pharmacy
Title: Bimolecular fluorescence complementation (BiFC) analysis of AP-1 dimierzation in living cells and *C. elegans*

09/28/10 Place: Nanjing University Medical School
Title: Multicolor bimolecular fluorescence complementation (BiFC): A novel high throughput screening method for protein-protein interactions

09/25/10 Place: Wannan Medical College
Title: Mechanisms and targeting of therapy-resistant prostate cancer

09/16/10 Place: Wuhan Institute of Virology
Title: Bimolecular fluorescence complementation (BiFC): Current Status and Future Perspectives

09/13/10 Place: Beijing University Cancer Hospital
Title: Mechanisms and targeting of therapy resistant prostate cancer

09/08/10 Place: Purdue University BIG Symposium
Title: Fluorescence complementation: An emerging tool for visualization of molecular events in living cells and animals

10/16/09 Place: Southern China Agriculture University

Title: Principle and applications of bimolecular fluorescence complementation (BiFC)

10/19/09 Place: Sun Yat-sen University Zhongshan Medical School
Title: Principle and applications of bimolecular fluorescence complementation (BiFC)

10/26/09 Place: Bengbu Medical College
Title: Principle and applications of bimolecular fluorescence complementation (BiFC)

10/28/09 Place: Nanjing University Medical School
Title: Seeing is believing: visualization of protein-protein interactions using bimolecular fluorescence complementation (BiFC),

05/07/09 Place: University of Chicago Graduate Program of Physiology
Title: Bimolecular fluorescence complementation (BiFC) analysis in living cells and living animals,

02/02/09 Place: Indiana University Medical School, Department of Biochemistry
Title: Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy

12/08/08 Place: University of Virginia Cancer Center
Title: Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy

11/25/08 Place: 7th International Conference on Photonics and Imaging in Biology and Medicine (Wuhan, China), Nov 24-27, 2008
Title: Fluorescence complementation: an emerging technology in biomedical research (presentation and panel discussion)

10/15/08 Place: 4th Modern Drug Discovery & Development Summit (San Diego, 15/10/08-17/10/08); Chair of Imaging Technology Symposium
Title: Multicolor fluorescence complementation in drug discovery

11/29/07 Place: UMDNJ-SOM Stratford
Title: Bimolecular fluorescence complementation analysis of AP-1 dimerization in living cells and living animals

11/28/07 Place: The Children's Hospital of Philadelphia and The University of Pennsylvania

Title: Molecular regulation and targeting of ATF2 nucleocytoplasmic shuttling

11/13/07 Place: Department of Biochemistry, Purdue University
Title: AP-1 biology, pathology, and technology

10/30/07 Place: Fluorescent proteins and Biosensors at HHMI Janelia Farm
Title: BiFC-FRET, a novel assay for visualization of ternary complexes in living cells (Invited for oral presentation)

08/07/07 Place: International Microscopy & Microanalysis 2007 at Ft. Lauderdale
Title: Bimolecular fluorescence complementation (BiFC) and beyond (Invited for oral presentation)

02/09/07 Place: Montana State University Department of Microbiology
Title: Functional analysis of AP-1 dimerization by bimolecular fluorescence complementation

11/01/06 Place: Vanderbilt University Institute of Chemical Biology
Title: Visualization of AP-1 protein interactions in living cells and in living animals using an improved BiFC system

10/04/06 Place: University of Illinois at Chicago School of Medicine
Title: Bimolecular fluorescence complementation: principle and applications

07/17/06 Place: Huazhong University of Science and Technology Tongji Medical College
Title: Bimolecular fluorescence complementation: principle and applications

03/14/06 Place: University of Toronto Western Research Institute
Title: Visualization of AP-1 protein interactions in living cells and in living animals using an improved BiFC system

09/30/05 Place: Eli Lilly, Indianapolis
Title: Identification of new fluorescent protein fragments for BiFC analysis under physiological conditions

03/10/05 Place: Purdue University, School of Health Science, Purdue University
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions

09/02/04 Place: Illinois State University, Department of Biology

Title: Role of *C. elegans* Fos and Jun homologs in development.

08/13/04 Place: Cold Spring Harbor (Cold Spring Harbor Image Course)
Title: Seeing is believing: visualization of transcription factor interaction in living cells and in living animals using a novel using bimolecular fluorescence complementation (BiFC) approach

05/07/04 Place: Purdue University, Department of Chemistry
Title: Seeing is believing: visualization of transcription factor interactions in living cells and in living animals

01/14/04 Place: Purdue University, Department of Biological Science
Title: Seeing is believing: visualization of transcription factor interactions in living cells and in living animals

12/04/03 Place: Indiana University at Bloomington, Department of Biology
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions

11/07/03 Place: Purdue Cancer Center (Purdue Cancer Center Director's Advisory council)
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions in cancer research

09/04/03 Place: Purdue Cancer Center (Annual Scientific Retreat)
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions

03/11/03 Place: Cincinnati Children's Hospital, Division of Experimental Hematology
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

03/04/03 Place: Harvard Medical School, MGH, Laboratories of Photomedicine
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

02/24/03 Place: Medical University of South Carolina, School of Pharmacy Department of Pharmaceutical Science
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

02/19/03 Place: University of Texas M.D. Anderson Cancer Center, Department of Molecular Therapeutics
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

02/06/03 Place: Ohio State University, School of Medicine Department of Physiology and Cell biology
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

12/28/02 Place: Purdue University Cancer Center
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells

07/20/00 Place: Bengbu Medical College, Bengbu, China
Title: Recent progress in the activation mechanisms of Raf by Ras

07/15/00 Place: Tongji Medical University, Wuhan, China
Title: Cloning and functional characterization of a novel type phospholipase C (PLC-ε)

Publications:

1. **Hu, C.D.**, Zhang, X.-H., and Bi, E.-H. Role of macrophages in the modulation of NK activity. *Foreign Medicine, Part of Immunology*, **10**, 16-20 (1987) (review in Chinese).
2. **Hu, C.D**. and Zhang, X.-H. Influence of EM on specific immune responses in normal Swiss mice. *Chinese Journal of Immunology*, **4**, 176-178 (1988) (in Chinese).
3. **Hu, C.D**. and Zhang, X.-H. Influence of EM on spleen cells NK activity and its mechanisms. *Chinese Journal of Microbiology and Immunol*ogy, **8**, 11-14 (1989) (in Chinese).
4. **Hu, C.D.**, Zhan, Z.-L., and He, S.-P. Study on the mutagenicity of trichloromethane. *Chinese J. Public Health*, **5**, 220-222 (1990) (in Chinese).
5. **Hu, C.D.**, Zhan, Z.-L. and He, S.-P. Study on the influential factors and the sensitivity of microtitre fluctuation test. *Journal of Healthy and Toxicology*, **4**, 115-118 (1990) (in Chinese).
6. **Hu, C.D.**, Kariya, K., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S., and Kataoka, T. Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *J. Biol. Chem*., **270**, 30274-30277 (1995).
7. Yanagihara, C., Shinkai, M., Kariya, K., Yamawaki-Kataoka, Y., **Hu, C.D.**, Masuda, T., and Kataoka, T. Association of elongation factor 1α and ribosomal protein L3 with the proline-rich region of yeast adenylyl cyclase-associated protein CAP. *Biochem. Biophys. Res. Commun*., **232**, 503-507(1997).
8. **Hu, C.D.**, Kariya, K., Kotani, G., Shirouzu, M., Yokoyama, S., and Kataoka, T.

Coassociation of Rap1A and Ha-Ras with Raf-1 N-terminal region interferes with Ras-dependent activation of Raf-1. *J. Biol. Chem*., **272**, 11702-11705 (1997).

9. Tamada, M., **Hu, C.D**., Kariya, K., Okada, T., and Kataoka, T. Membrane recruitment of Raf-1 by association is not only the major function of Ras in Raf-1 activation, *Oncogene*, **15**, 2959-2964 (1997).
10. Shibatohge, M., Kariya, K., Liao, Y**., Hu, C.D.,** Watari, Y., Goshima, M., Shima, F., and Kataoka, T. Identification of PLC210, a *C. elegans* homolog of phospholipase C, as a putative effector of Ras, *J. Biol. Chem*., **273**, 6218-6222 (1998).
11. Shirouzu, M., Morinaka, K., Koyama, S., **Hu, C.D**., Hori-Tamura, N., Okada, T., Kariya, K., Kataoka, T., Kikuchi, A, and Yokoyama, S. Interactions of the amino acid residue at position 31 of the c-Ha-Ras with Raf-1 and RalGDS, *J. Biol. Chem*., **273**, 7737-7742 (1998).
12. Ohnishi, M., Yamawaki-Kataoka, Kariya, K., Tamada, M., **Hu, C.D**., and Kataoka, T. Selective inhibition of Ras interaction with its particular effector by synthetic peptides corresponding to the Ras effector region, *J. Biol. Chem*., **273**, 10210-10215 (1998).
13. Kataoka, T., Kariya, K., Yamawaki-Kataoka, Y., **Hu, C.D**., Shirouzu, M., Yokoyama, S., Okada, T., and Shima, F. Isoprenylation-dependent and independent interaction of Ras with its effectors. In Kuzumaki, N. Cytoskeleton and G-Protein in the Regulation of Cancer. *Hokaido University Medical Library Series*, **37**, 141-146 (1998).
14. Watari, Y., Kariya, K., Shibatohge, M., Liao, Y., **Hu, C.D**., Goshima, M., Tamada, M., Kikuchi, A., and Kataoka, T. Identification of Ce-AF-6, a novel *Caenorhabditis elegans* protein, as a putative Ras effector, *Gene*, **224**, 53-58 (1998).
15. **Hu, C.D**., Kariya, K., Okada, T., Qi, X., Song, C., and Kataoka, T. Effect of phosphorylation on activities of Rap1A to interact with Raf-1 and to suppress Ras-dependent Raf-1 activation , *J. Biol. Chem*., **274**, 48-51 (1999).
16. Okada, T., **Hu, C.D**., Jin T.-G., Kariya, K., Yamawaki-Katatoka, Y., and Kataoka, T. The strength of interaction at the Raf cysteine-rich region domain is a critical determinant of response of Raf to Ras family small GTPase. *Mol. Cell. Biol.* **19**:6057-6064 (1999).
17. Tanaka, Y., Minami, Y., Mine, S., Hirano, H., **Hu, C.D**., Fujimoto, H., Fujii, K., Saito, K., Tsukada, J., van Kooyk, Y., Figdor, C. G., Kataoka, T., and Eto, S. H-Ras signals to cytoskeletal machinery in induction of integrin-mediated adhesion of T cells. *J. Immunol*., **163**, 6209-6216 (1999).
18. Liao, Y., Kariya, K**., Hu, C.D**., Shibatohge, M., Goshima, M., Okada, T., Watari, Y., Gao, X., Jin, T.-G., Yamawaki-Katatoka, Y., and Kataoka, T. RA-GEF, a novel Rap1A guanine nucleotide exchange factor containing a Ras/Rap1A-associating domain, is conserved between nematode and humans. *J. Biol. Chem*. **274**, 37815-37820 (1999).
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